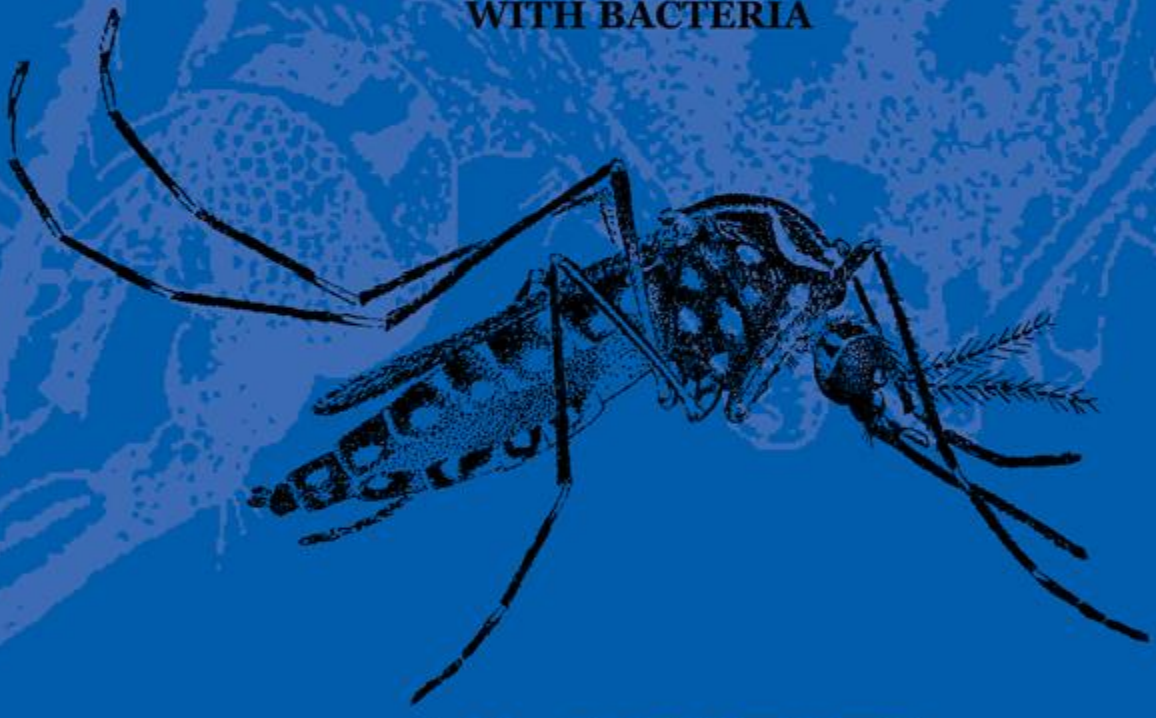
THE BIOLOGY OF MOSQUITOES

VOLUME 3

TRANSMISSION OF VIRUSES AND INTERACTIONS WITH BACTERIA



A.N. CLEMENTS

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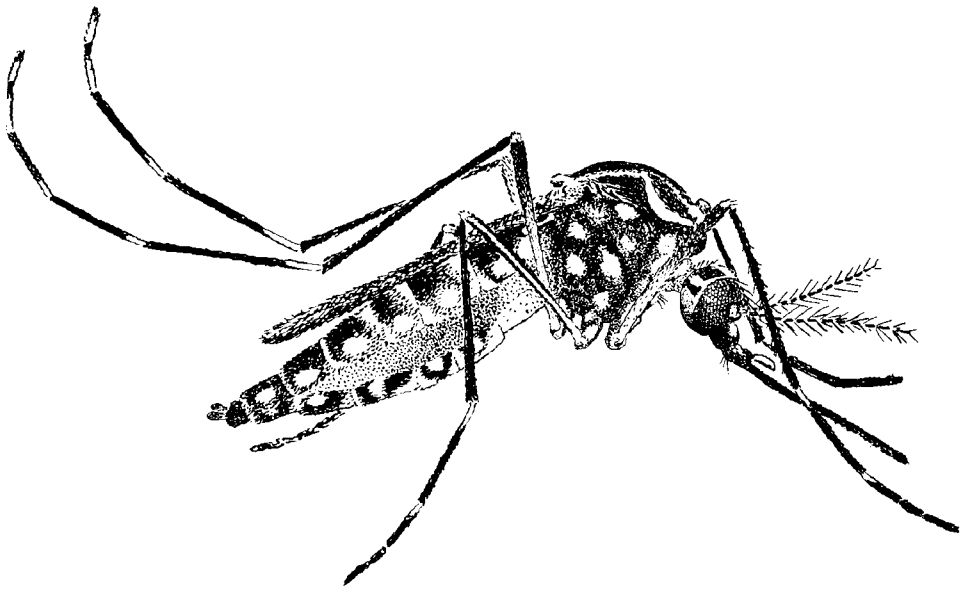
TRANSMISSION OF VIRUSES AND INTERACTIONS WITH BACTERIA

A.N. CLEMENTS

London School of Hygiene and Tropical Medicine



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Preface

An intended single volume to have been titled *The Biology of Mosquitoes* expanded into two volumes, published in 1992 and 1999, which broadly concerned mosquito physiology and mosquito behaviour, respectively. A chapter on the pathogens and parasites of mosquitoes inserted into the projected third volume expanded so greatly that it alone came to constitute this third volume, on the transmission of viruses and interactions with bacteria, plus a fourth on the transmission of unicellular and multicellular parasites, now partly written. The projected fifth volume is more distant.

An important development in this volume is adoption of the revised, phylogenetic classification of mosquitoes of the tribe Aedini. Cladistic analysis of morphological data makes it possible to determine the evolutionary relationships of organisms and to produce phylogenetic classifications. This scientific advance has been adopted in recent decades by the taxonomists of most groups of living organisms, including insects, but, curiously, not by most mosquito taxonomists. Over recent years a phylogenetic classification of the culicid tribe Aedini was developed which regroups them into smaller genera with more distinct geographical distributions and, in some cases, distinctive biology – developments that have been invaluable in assembling and rationalizing a multitude of data for this review. This was most obvious in the division of the traditional genus *Aedes* – which has well over 900 species – into its phylogenetically distinct lineages. Because this advance in mosquito biology appears to be unknown to many, a description and justification are provided in the Introduction which immediately follows this Preface. The changes to generic names that resulted from the reclassification are detailed in Appendix 2.

Alan Clements

Introduction

The traditional and revised classifications of aedine mosquitoes

A recent phylogenetic revision of the classification of the tribe Aedini necessarily led to changes in the nomenclature of that group, introducing a number of restored or newly designated genera. Where they are relevant, those genus names are used in this volume. Initial responses to the changes of classification and nomenclature have generally not been welcoming, so it seems sensible, at the beginning of the volume, to contrast the traditional and revised classifications, and to describe the scientific reasons for adoption of the revised classification and the practical advantages that it provides.

THE TRADITIONAL CLASSIFICATION OF MOSQUITOES

By the end of the 19th century much was known of the biology of mosquitoes, and detailed descriptions had been published of their life-cycle stages, adult feeding mechanisms, oviposition behaviour, internal anatomy and histology. A classification had been developed, but based on only a small proportion of the species that are now known. Discovery during the last two decades of the 19th century of the role of mosquitoes in the transmission of microfilariae and malaria parasites triggered a drive to collect mosquitoes wherever possible and to name and classify them. New species were described and many new genera designated by mosquito taxonomists, notably Blanchard and Neveu-Lemaire in France, Theobald in England, and Dyar and Knab in the United States. A Committee set up by The Royal Society in 1899 to inquire into the causes and control of malaria appointed Theobald to prepare a monograph on the mosquitoes of the world, and this was published between 1901 and 1910 in five volumes (Theobald, 1901–1910). In North America, Howard, with Dyar and Knab, published reviews of the mosquitoes of North and Central America in four volumes between 1912 and 1917 (Howard *et al.*, 1912–1917).

It was a feature of these early taxonomic studies that genera and higher taxa were distinguished on the basis of rather few characters. Theobald (1901a, 1901–1910) defined genera very largely on the shape and arrangement of scales on the adults and, despite early criticism, continued to do so. In a survey of culicid larvae, Dyar and Knab (1906) distinguished the subfamilies Anophelinae, Culicinae and Sabethinae, and their genera and species, solely on larval characters. Dyar (1918) used the characteristics of male genitalia to distinguish some culicid genera. Possibly due to such simplifications, certain taxonomists discarded many previously designated genera, listing each as a junior synonym of a related genus that had historical precedence. As early as 1906, Dyar and Knab subsumed 16 genera into *Anopheles*, 13 into *Aedes* and five into *Culex*; later, Dyar (1922) subsumed 37 genera into *Anopheles*, 49 into *Aedes* and 33 into *Culex*.

F.W. Edwards succeeded Theobald at the British Museum and contributed further to mosquito taxonomy; in 1932 he published a revised classification of the family Culicidae. The genera *Anopheles*, *Culex* and *Aedes* remained very species rich, while many of the early genera that had been put aside as junior

synonyms were given the rank of subgenus in one or other of them. In the introduction to the 1932 work, Edwards described 'the advantages of employing larger generic concepts', asserting that: (i) the wider relationships of the species are more clearly indicated; (ii) limits can be more readily assigned to large genera than to more numerous, smaller groups; (iii) avoidance of the duplication of specific names is ensured; (iv) generic diagnoses should be applicable to both sexes; and (v) the use of subgeneric terms enables those who wish to do so to make use of the smaller divisions. Most of these 'advantages' concerned expediency rather than taxonomic correctness and, today, with our much greater knowledge of mosquitoes, some of the supposed advantages are no longer pertinent.

Edwards (1932) retained the taxa Dixinae and Chaoborinae in the Culicidae as subfamilies, together with the so-called true mosquitoes – the subfamily Culicinae, with its 30 genera, 89 subgenera and 1400 species. With some major alterations, i.e. the removal of the subfamilies Dixinae and Chaoborinae, and the designation of the subfamilies Anophelinae, Culicinae and Toxorhynchitinae, Edwards' classification was adopted in Stone *et al.*'s (1959) *A Synoptic Catalog of the Mosquitoes of the World*. The last major restructuring of the traditional classification was in Knight and Stone's (1977) *A Catalog of the Mosquitoes of the World*, in which the subfamily Culicinae was divided into ten tribes, one being the tribe Aedini. Each of the nine genera within the Aedini consisted of one to three subgenera except for the genus *Aedes*, which had 38 subgenera. The growth in number of species since 1977 and the designation of further genera are recognized now in the 'Systematic Catalog of Culicidae' (Gaffigan *et al.*, 2011), which is compiled and maintained online by the Walter Reed Biosystematics Unit (WRBU; http://wrbu.si.edu/) of the Walter Reed Army Institute of Research (WRAIR) in Maryland.

CRITICISMS OF THE TRADITIONAL CLASSIFICATION OF MOSQUITOES

What are now perceived as defects of the traditional classification of mosquitoes arose partly because it was produced before the development of modern taxonomic techniques. To some extent, phenetic methods that rely on estimates of overall similarity were used; but, as Black (2004) pointed out, the morphological characters that taxonomists used in dichotomous keys to identify species were also used to classify species into higher taxonomic groups. Consequently, while certain genera are monophyletic, significant numbers of genera are paraphyletic or polyphyletic.

Other problems arose because of the preference for large genera. Bates (1949) pointed out that, whereas Theobald (1901–1910) had grouped 1050 species into 149 genera, Edwards (1932) had grouped 1400 species into only 30 genera. Belkin (1962) commented that 'Many of the subgenera of *Aedes* appear to be heterogeneous complexes of superficially similar species, and it is very probable that they will have to be subdivided into smaller natural groups', and argued that 'Lumping all small taxa into groups of a convenient size but of indefinite affinities does nothing but obscure relationships'. He concluded that 'The internal classification of the Aedini is in need of thorough revision'. Tanaka *et al.* (1979) described *Aedes* as 'A polymorphic genus; most characters extremely variable'.

The situation was given perspective when Zavortink (1990) contrasted the traditional classification of the Culicidae with the classifications typical of most other groups of organisms. A number of authorities, including Williams (1951), Dial and Marzluff (1989), Mayr and Ashlock (1991) and Scotland and Sanderson (2004), had shown that a 'hollow curve' distribution is shown graphically when the number of known species per genus is plotted against the number of genera with the corresponding number of species (Figure I.1A). Generally, taxa of family rank consist of a number of monotypic (single species) genera, a smaller number of ditypic genera, and progressively fewer genera at each further increment in number of species per genus, culminating in the very few genera that are relatively species rich. Hyperbola-like or hollow-curved distributions have been documented for higher taxa of plants, crustaceans, insects, fish, birds and

mammals. They were obtained whether cladistic analyses or other methods had been used to develop the classifications, and whether the taxonomists had been 'lumpers' or 'splitters' of species into genera.

By comparing the species richness of culicid genera with that of other groups of organisms, Zavortink (1990) showed that the family Culicidae consisted of far fewer genera than did most families with a similar number of species. Instead of a hollow curve, a plot of numbers of culicid genera against their species richness yielded an almost flat line along the X axis (Figure I.1B). By comparison with the classifications of other organisms, Zavortink calculated that the 3146 mosquito species then recognized should have been grouped into about 225 genera, not 37. The number of culicid genera was many fewer than would be expected for a family that had achieved the beta level of taxonomic investigation (i.e. the level of arrangement of species into hierarchical systems of higher categories or taxa). Zavortink concluded that 'we have not even begun to develop a natural classification for the family'. This analysis had no influence on mosquito taxonomists, and 20 years later the three largest culicid genera in the accepted classification were still exceedingly large, with *Anopheles* comprising *c.* 475 species in seven subgenera, *Culex c.* 793 species in 27 subgenera and *Aedes c.* 927 species in 45 subgenera (data from the online Systematic Catalog of Culicidae, Gaffigan *et al.*, 2011).

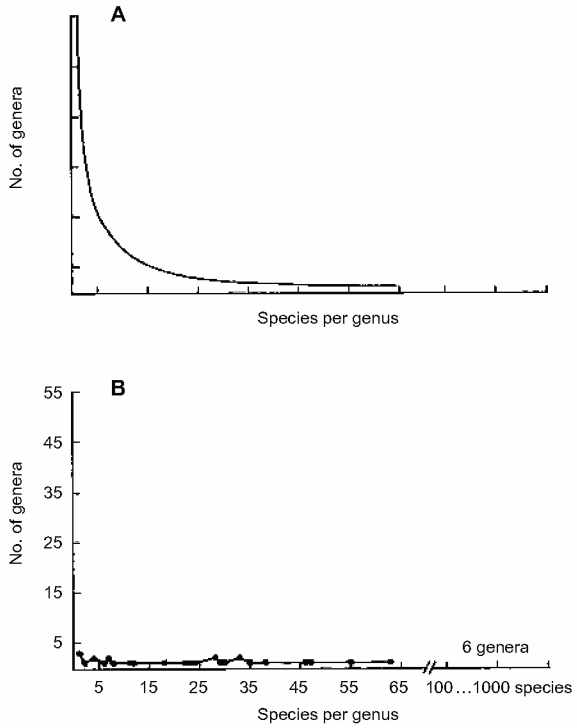

Figure I.1 (A) Hollow curve showing the decrease in the number of genera as the number of species per genus increases for any group of organisms with a sound natural classification. (B) Number of mosquito genera (Culicidae) with the number of species indicated. (Figures after Zavortink, 1990, with original captions.) The upper figure showing a hollow curve was based on many published findings. In the lower figure from data for the family Culicidae in its traditional classification, the plot takes the form of an almost straight line close to the X axis.

PHYLOGENETIC CLASSIFICATIONS BASED ON CLADISTIC ANALYSES

Classifications of animal groups that have been developed from cladistic analyses of morphological data reflect evolutionary relationships and are phylogenetic. Hennig (1950, 1966) redefined and clarified the concepts of monophyly and of phylogenetic relationships, and introduced cladistic techniques that sought patterns of similarity based only on shared evolutionarily novel features. Phylogenetic hypotheses were developed from which the basis of a classification could be developed using monophyly, and the principle of equivalent rank used as the criterion for recognizing and grouping taxa. A phylogenetic classification had been achieved when all taxa were monophyletic and each subsidiary taxon had evolved from a single ancestor. During the three decades that followed Hennig's contributions, the theory of phylogenetic systematics was refined and cladistic methods were improved (Kitching *et al.*, 1998).

Phylogenetic classifications are now almost universally accepted as the most useful general reference system for biology. Andersen (2001) observed that the 'higher' Diptera now have a natural classification, whereas the more primitive nematoceran families, including the Culicidae, are in need of cladistic, phylogenetic analysis.

DEVELOPMENT OF A PHYLOGENETIC CLASSIFICATION OF AEDINE MOSQUITOES

In an early study, using characters of the female and male genitalia, and supplementary characters of larvae and pupae from over 65% of known species of *Aedes*, Reinert (2000) divided the composite genus *Aedes* into two genera: genus *Aedes* (with 22 subgenera) and genus *Ochlerotatus* (with 21 subgenera). That first step led on to cladistic analyses of the Aedini, the largest tribe in the family Culicidae, and involved examination of a high proportion of known aedine species. The ranking of lineages as genera or subgenera involved the criteria of monophyly and equivalent rank. As the investigation progressed through four phases, the findings were published by Reinert *et al.* in 2004, 2006, 2008 and 2009.

The first phase assessed 119 exemplar species representing the 12 traditionally recognized aedine genera and 56 subgenera, with examination of 172 characters from all life-cycle stages. Cladistic analyses yielded eight most parsimonious cladograms from which a strict consensus tree was constructed that permitted two possible phylogenetic classifications of the tribe Aedini. The principal problem was not in recognizing monophyletic groups, but in deciding what taxonomic ranks should be assigned to them once their phylogenetic relationships had been established. In one of the two possible classifications, the 11 traditionally accepted genera, i.e. *Aedes* (inclusive of *Ochlerotatus*), *Armigeres*, *Ayurakitia*, *Eretmapodites*, *Haemagogus*, *Heizmannia*, *Opifex*, *Psorophora*, *Udaya*, *Verrallina* and *Zeugnomyia*, would all be subsumed into the genus *Aedes*, which would become the sole genus of the tribe Aedini. In the second and preferred possible classification, all clades having phylogenetic equivalence with the 11 traditionally accepted genera would have the rank of genus. With the restoration of many subgenera to their former rank and the designation of new genera, the total number of genera would be raised to 46. A provisional, revised classification of aedine mosquitoes was proposed on that basis (Reinert *et al.*, 2004).

In the later phases, the investigation was extended to aedine groups that had not been examined in detail earlier. The second phase concerned the genus *Finlaya* and associated taxa, and involved examination of 232 characters in 116 exemplar species (Reinert *et al.*, 2006). The third phase concerned *Ochlerotatus* and associated taxa, and involved examination of 297 characters from 159 exemplar species (Reinert *et al.*, 2008). In the final phase, the phylogeny and classification of the tribe Aedini were delineated based on a cladistic analysis of 336 characters from eggs, 4th instar larvae, pupae, and adult females and males from 65 genera and 46 subgenera, habitat coded for 270 exemplar species (Reinert *et al.*, 2009). The investigation resulted in the recognition of 80 genera and 48 subgenera within the tribe Aedini, all of which, with the exception of the genus *Dendroskusea* Edwards, were recovered as monophyletic taxa. As was usual with

groups of Aedini at the generic level, all newly recognized genera and subgenera were polythetic taxa, i.e. they were diagnosed by unique combinations of characters, none of which was diagnostic alone.

An updated, phylogenetic classification of the Aedini is available online in the Mosquito Taxonomic Inventory (Harbach, 2011, http://mosquito-taxonomic-inventory.info/). Within the Inventory, a list headed Resources includes two pertinent items. (i) The item 'Aedini Classification' provides a pdf file (of that name) in which all aedine genera and subgenera of the revised classification are listed, in parallel with equivalent taxa of the traditional classification. (ii) The item 'Valid Species List' provides a pdf file (of that name) in which the names of all valid mosquito species are recorded.

CRITICISMS MADE OF THE REVISED CLASSIFICATION OF AEDINE MOSQUITOES

The initial division of the composite genus *Aedes* into the genera *Aedes* and *Ochlerotatus* by Reinert (2000) was opposed by Savage and Strickman (2004), who argued that 'Use of these names as genera complicates mosquito identification and interferes with information retrieval among taxonomists, medical entomologists, and vector control specialists'.

The *Journal of Medical Entomology* (*JME*, 2005; vol. 42, p. 511) carried a 'Letter from the Editor' entitled 'Journal Policy on Names of Aedine Mosquito Genera and Subgenera'. The 'Letter' started by citing, but rephrasing, a paragraph from the Preamble to the International Code of Zoological Nomenclature. The original paragraph reads: 'The objects of the Code are to promote stability and universality in the scientific names of animals and to ensure that the name of each taxon is unique and distinct. All its provisions and recommendations are subservient to those ends and none restricts the freedom of taxonomic thought or actions'. Referring to that statement, the 'Letter' asserted that 'When reinterpreting the relationships between species and groups of applied importance, systematists have responsibility to limit the impact on nomenclature. Such caution was not exercised when the majority of known species and subgenera of *Aedes* mosquitoes were transferred to the restored genus *Ochlerotatus* based on taxonomic characters that few other workers have examined (Reinert, 2000)'. That opinion was expressly refuted by Polaszek (2006) in an article published from the office of the International Commission on Zoological Nomenclature.

Publication of a first cladistic analysis and proposed phylogenetic classification of the tribe Aedini by Reinert *et al.* (2004), with the restoration of some former genera and the designation of new genera, prompted further criticism from Savage (2005), who considered that 'The entire approach to Aedini systematics of these authors was flawed by an inordinate fear of paraphyletic taxa, or paraphylyphobia, and their inability to distinguish between classification and cladistic analysis'. Referring to the cladistic analyses of Reinert *et al.* (2004), the 'Letter' from the Editor in *JME* (2005) discouraged submission to that journal of articles using the revised nomenclature, and reported support from the editors of several other journals of medical entomology or tropical medicine. A virtually identical article was published in the *American Journal of Tropical Medicine and Hygiene* (Weaver, 2005). That prohibitive attitude has persisted in certain journals up to this time.

Considered and realistic criticism of Reinert *et al.* (2004) appeared online in a WRBU Forum which had been established by a Mosquito Systematics Review Committee. Unfortunately, the Forum is no longer accessible. The key criticisms, set out in the 'Summary of majority opinion' of the Committee, mostly concerned technical aspects of the cladistic techniques that had been used and the interpretation of data, matters to which I.J. Kitching published a detailed response in the Forum proceedings. Following its deliberations, the Review Committee rejected Reinert *et al.*'s (2004) proposed classification of the Aedini in the WRBU online Systematic Catalog of the Culicidae.

The critics could have found their comments and criticisms partly explained, or even agreed with, in the following lines from the brief 'Final Comments' section in Reinert *et al.*'s (2004) 88-page article: 'The lack of basal resolution and branch support in the cladograms makes it impossible to fully resolve the

relationships among the genera ... . Additional morphological data may provide a clearer view of the relationships between closely related genera, but are unlikely to resolve deeper relationships within the tribe. ... The next step will be to resolve the relationships and placement of taxa of uncertain taxonomic position to achieve a more robust classification ...'. The first part of that last sentence was the only indication that this article covered only the first phase of what was to become a very extensive investigation.

It might be taken as affirmation of the validity of the phylogenetic classification of the tribe Aedini that no criticisms were published of the reports from the later phases of the investigation (i.e. from Reinert *et al.*, 2006, 2008, 2009). The taxon *Ochlerotatus* was accepted as having genus rank, possibly due to its importance in North America. That only few entomologists in North America and Western Europe used the remainder of the revised nomenclature in journal articles probably reflected not only the refusal of journal editors to accept them but also a widespread ignorance of this development in taxonomy. Elsewhere, however, the revised classification became adopted, as in important publications from China, Vietnam, Thailand, Iran and Saudi Arabia.

No keys have been published that lead investigators to the newly named genera. However, in earlier taxonomic articles that used the traditional nomenclature of aedine mosquitoes, keys to the traditional subgenera of *Aedes* lead to taxa that now have genus rank, while keys to the species of the traditional subgenera of *Aedes* that have been restored to or raised to genus rank will enable users to direct those species to the new genera.

The hostility that met the change of the scientific name of the yellow fever mosquito, from *Aedes* (*Stegomyia*) *aegypti* L. to *Stegomyia aegypti* L., is ironic. The mosquito to which Linnaeus assigned the name *Culex aegypti* in 1792 was a species of *Ochlerotatus*, and definitely not the yellow fever mosquito (Gough, 1914; Patton, 1933). Earlier, mosquitoes used in the experimental transmission of yellow fever virus had been given the valid name *Aedes* (*Stegomyia*) *fasciata* (Fabricius) (Howard, 1901). The name *aegypti* L. must now be applied to the yellow fever mosquito instead of *fasciata* (Fab.) only because, at the request of medical entomologists, the International Commission on Zoological Nomenclature (1964) used its plenary powers to validate the specific name *aegypti* Linnaeus, to be interpreted by reference to a neotype, being a specimen of the yellow fever mosquito from Malaya (now peninsular Malaysia).

PRACTICAL DIFFICULTIES THAT FOLLOW ADOPTION OF THE PHYLOGENETIC CLASSIFICATION

From the first, critics correctly pointed out that the introduction of a phylogenetic classification, with many new generic names, would cause problems with information transfer. To quote just one critic, it would 'inevitably create considerable confusion among teachers, students and researchers, with communication difficulties and financial implications for republishing educational materials, keys, catalogues and management of data bases'. In reality, the greatest medium- to long-term problems lie in the retrieval of past information on those binomials in which the genus name has been changed, with an additional problem where the change of genus name required a change of gender of the species name. The problem is less great where the new genus names result from the restoration of subgenera to genus rank. It should not be overlooked that, to a degree, this nomenclatural problem resulted from the practice of subsuming many mosquito genera into a very few.

Interestingly, in his book *The Natural History of Mosquitoes*, Marston Bates (1949) had stated 'The (other) cause of name changing results from the discovery of new relationships, of new and perhaps more satisfactory methods of classifying a group of animals. Rearranging these animals under different generic concepts may cause temporary inconvenience, but if the end result is an improved classification the inconvenience is a small cost'.

In this volume, a high proportion of the species that are cited with genus names different from those in the traditional nomenclature are species now assigned to *Ochlerotatus*, a genus that has been widely accepted for some years. Another frequent change concerns the yellow fever mosquito, with a change of scientific name from *Aedes* (*Stegomyia*) *aegypti* to *Stegomyia aegypti*. If these provide any measure of the effects of adoption of the new nomenclature more generally, then the supposed difficulties have been overemphasized.

BIODIVERSITY

Life is more abundant in the tropics than towards the poles. Over each hemisphere a 'latitudinal diversity gradient' is apparent as a progressive increase in the numbers of species and of higher taxa from the polar regions to the tropics. It has been postulated that, for the most part, taxa originate in the tropics and that, while persisting there, extend their distribution over time into higher latitudes. If that is the case, then the numbers of endemic genera should be greater at the lower latitudes (Jablonski *et al.*, 2006).

Analysis of the global distribution of culicids revealed a latitudinal diversity gradient of species richness. A frequency curve produced by plotting the mean number of mosquito species per km^2 of the countries intersected by bands of 10° of latitude, and extending across the northern and southern hemispheres, revealed a progressive decline in species richness in each hemisphere as the bands of latitude moved away from the equator and towards the poles (Figure I.2). Excluding small island countries, the countries with the largest numbers of total mosquito species, and of endemic mosquito species, were Panama, French Guiana, Malaysia and Costa Rica, all tropical countries that are situated between 1° 2' N and 11° 12' N (Foley *et al.*, 2007).

The causes of the latitudinal diversity gradient remain a matter of conjecture, but it is possible to discern changes of environmental structure with change of latitude which could affect the extent of biodiversity. One is the greater spatial heterogeneity apparent in the tropics, which is associated with a greater variety of microenvironments. Further, it is supposed that the greater seasonal stability of tropical environments has led to the evolution of more specialized species with narrower ecological niches (Calow, 1998). Culicids occur in a variety of habitats, most distinctly during their aquatic stages, when their habitats range from bodies of open water of a variety of forms to container habitats. In the subtropics and tropics, culicid larvae

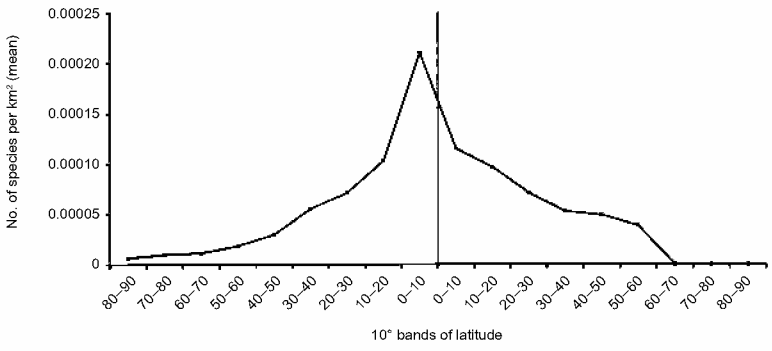

Figure I.2 Latitudinal diversity gradients of culicid species over the northern and southern hemispheres. The vertical line indicates the equator. The frequency curve shows the variation in species richness of culicids with latitude, as expressed by the mean number of culicid species per km^2 of countries intersected by bands of 10° of latitude. Northern hemisphere, left; southern hemisphere, right. (The figure was generously provided by Dr Desmond Foley, who had calculated the values of the data points from country records in the Systematic Catalog of the Culicidae, Gaffigan *et al.*, 2011.)

make greater use of phytotelmata, including leaf axils, floral bracts, tank bromeliads, the pitchers of pitcher plants and bamboo internodes, and also of natural cavities, including rock holes, swamp crypts, animal footprints, crab holes and mollusc shells.

For any area or geographic region, interest in biodiversity extends beyond species richness to the taxonomic identity of the species and the ecological niches that they occupy. The intensive studies that have been undertaken on mosquito species and their ecology have made an exceptional contribution to the knowledge of biodiversity in many countries. However, studies of biodiversity can be seriously disadvantaged if the taxonomic classifications are not phylogenetic. Concealed within the genera and subgenera of the traditional classification of mosquitoes are groups of species that cladistic analyses show to be distinct, monophyletic lineages which have distinct distributions and biological characteristics. Consider *Danielsia*, *Hulecoetomyia* and *Phagomyia*, genera designated by Theobald, but which in the traditional classification are treated as junior synonyms of *Aedes* (*Finlaya*). Cladistic analysis showed them to be distinct lineages meriting the rank of genus. It also revealed new monophyletic lineages within *Aedes* (*Finlaya*) that warranted genus rank (Reinert *et al.*, 2009).

END NOTE

This Introduction, in its original form as a longer review article, was rejected on principle by a leading journal of medical entomology, but since then attitudes to the phylogenetic classification and nomenclature of aedine mosquitoes have started to change. The same journal now welcomes articles that use the revised nomenclature; articles of regional mosquito taxonomy that use the revised nomenclature have been published in China, Korea, Thailand, Iran and Saudi Arabia; and a leading post-graduate institution in the United Kingdom teaches the revised nomenclature solely.

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41

Host/parasite interactions

41.1 CHARACTERISTICS OF INFECTIOUS AGENTS, THEIR VERTEBRATE HOSTS AND MOSQUITO VECTORS

41.1.1 Characteristics of infectious agents

Terms associated with infection and parasitism may be defined and used rather differently by writers with different interests, e.g. by parasitologists and ecologists, so it is necessary to describe how they are used in this volume (see also Glossary, Appendix 3). The most basic term, **infectious agent**, embraces both viruses and cellular organisms. The term **infectious organism** applies to infectious micro-organisms (bacteria and unicellular eukaryotes) and infectious multicellular eukaryotes. Infectious agents may be classed as pathogens or parasites, two terms that are used interchangeably by some authorities but are distinguished as apart by others. Here, the term **pathogen** is applied to viruses and infectious bacteria that adversely affect their hosts, while the term **parasite** is applied to infectious eukaryotes, whether unicellular or multicellular, that adversely affect their hosts. The adjectival forms of those two terms are used less restrictively, even to the extent of overlapping usage. For example, the term **pathogenic** is applied to the deleterious effects of both pathogens and parasites, and the term **parasitic** may be applied to the infectious lifestyle of both pathogens and parasites.

Pathogens may be classed as obligate (facultative) or opportunistic. **Obligate** pathogens live in intimate association with their hosts, and their long-term survival depends on their ability to replicate in the host. **Opportunistic** pathogens do not require intimate association with their hosts and can survive away from a host.

During the periods of their evolution, infectious agents have exploited some of the many mosquito/vertebrate associations in which particular mosquito species feed on particular vertebrate species. They undergo appropriate life-cycle phases alternately in the mosquito and vertebrate and, in consequence, the mosquitoes function as biological vectors. The infectious agents for which mosquitoes are biological vectors are: (i) viruses of five families; (ii) some species of *Hepatozoon* (class Coccidea); (iii) some species of *Plasmodium* (class Hematozoea); and (iv) species of a number of genera of Onchocercidae (superfamily Filarioidea).

Parasites may be grouped in two categories – microparasites and macroparasites – which are distinguished not just by size but by a number of biological characteristics (Anderson, 1979; Anderson and May, 1991). **Microparasites** undergo asexual reproduction within a host, usually at very high rates. They tend to be small and to have a short generation time. They are often intracellular. Infections of invertebrates by microparasites

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frequently prove fatal, but not when that host also serves as a vector. Vertebrate hosts that recover from infection by microparasites have usually developed immunity against reinfection, often for life. Usually, the duration of infection is short relative to the average lifespan of the vertebrate host, so for the survivors most microparasitic infections are transient. For that reason, the population growth of microparasites that infect vertebrates requires recruitment of susceptible individuals to the host population. Most obligate bacterial pathogens have the characteristics of microparasites. Many viruses and protozoan and fungal pathogens share these characteristics, but less completely.

Macroparasites undergo sexual but not asexual reproduction within the definitive host. Typically, they are larger than microparasites and are usually extracellular. They grow within the host, and their generation times are relatively long, frequently lasting for an appreciable fraction of the host's life-span. Their transmission stages (eggs or larvae) are often produced in very large numbers, and either pass into the external environment or are ingested by vectors. The immune responses of vertebrates to macroparasites tend to be of a relatively short duration. Macroparasite infections typically are persistent, with hosts being continually reinfected. The growth of infrapopulations within individual hosts results from the gradual accumulation of new infections. Relatively few macroparasites sequentially infect invertebrate and vertebrate hosts; among these, digeneans and some cestodes undergo asexual reproduction in the intermediate host. Acanthocephalans, parasitic platyhelminths, nematodes and arthropods have the characteristics of macroparasites.

41.1.2 Characteristics of different types of vertebrate host

Some pathogens or parasites sequentially infect two different hosts during a life cycle, and those hosts are functionally distinguishable by the uses made of them by the infectious agent. A **definitive host** is one in which an infectious organism lives for part of its life cycle and in which it attains sexual maturity. An **intermediate host** is one in which the infectious organism lives for part of its life cycle, and in which either it does not become sexually mature or only the asexual stages occur. Species that are susceptible to infection by a particular pathogen fall into one or other of two categories – competent and incompetent. When infected, **competent hosts** develop a viraemia or parasitaemia that is of sufficient titre and duration to infect blood-feeding vectors in the natural habitat. When infected, **incompetent hosts** do not develop such a viraemia or parasitaemia, and in the natural habitat cannot infect blood-feeding vectors. In practice, the term 'competent host' is now often replaced by **amplifying host**, and the term 'incompetent host' is replaced by **dead-end host** (Section 44.2.2).

The causative agents of some diseases of wild animals can be transmitted to humans, and such diseases are termed **zoonoses**. The species from which infectious agents can be transmitted must be competent hosts that generate a parasitaemia or viraemia of sufficient magnitude and duration to permit infection of vectors. The best single indicator of the involvement of a vertebrate species in a transmission cycle is frequent isolation of the infectious agent from free-ranging individuals. From serological evidence, it is known that Rift Valley fever virus (RVFV) infects mammals of a number of orders (Section 45.6.2.a). However, seropositivity for RVFV indicates no more than that an individual has been infected, and for only very few species is there virological evidence that indicates whether they could be amplifying hosts of RVFV. For effective transmission from wild hosts to humans, the vector species must be abundant and must feed frequently on both the wild host and on humans.

Infections of host populations vary in their epidemiological characteristics, but usually may be classed as enzootics, epizootics or epidemics. An **enzootic** is a disease that is constantly present in (wild) animal populations within a specified area, and usually at a low rate of prevalence. Virulence to the host species is generally but not necessarily

low, and the host and pathogen populations coexist in a balanced state; consequently the disease is always present. An enzootic system can become epizootic through a change in one or more of the key components – host, pathogen or environment.

An **epizootic** is an outbreak of an infectious disease in a (wild) animal population that is characterized by an exceptionally large number of cases relative to the number occurring earlier. Epizootics are sporadic and limited in duration, show sudden changes in incidence and then prevalence, and are usually caused by pathogens that have a short generation time. The change in prevalence depends first on massive reproduction of the causal organism, reinforced by environmental and host factors. At the beginning of an epizootic the host population must largely be susceptible to infection by the causal agent. When the proportion of susceptible hosts has fallen sufficiently, the high multiplication rate of the causal agent declines (Fuxa and Tanada, 1987). The term **epornithic** may be used for an outbreak of an infectious disease in a wild bird population. An **epidemic** is the equivalent of an epizootic but occurs in a human population or, as used by some authors, among domesticated livestock.

41.1.3 Characteristics of mosquito vectors

An individual infectious agent might be transmitted by one, a few or many species of mosquito. Within a zoogeographical region, a number of mosquito species might show **vector competence** for an infectious agent in the laboratory, i.e. susceptibility to infection and permissiveness for replication of that agent and its transmission in saliva. Not all species that show vector competence in the laboratory are vectors in the field. Other factors are important, including sympatry with the vertebrate host species, co-occurrence with the host species in time, and an appropriate host preference of the adult females. Species with vector competence that display these other characteristics are said to have **vectorial capacity**. (See also Glossary, Appendix 3)

A number of terms have been coined to distinguish between species whose capabilities as vectors differ quantitatively or in other ways. The terms 'primary vector' and 'secondary vector' are flawed (Smith, 1967), and the following terms, proposed by White (1982), are used here. A **main vector** is a widespread species capable of maintaining transmission of a particular infectious agent. In some locations there can be more than one main vector, but with differences in seasonality, ecology or behaviour. Thus, in parts of Africa where *Anopheles gambiae* s.l. and *Anopheles funestus* are sympatric, both can be main vectors of *Plasmodium falciparum*, but *An. funestus* becomes the more important when the larval habitats of *An. gambiae* are flushed out by prolonged heavy rains. A **subsidiary vector** will be one of three types – incidental, local or bridge. (i) An 'incidental vector' is a species that is incapable of maintaining endemicity on its own but that regularly supplements transmission where it is sympatric with a main vector. (ii) A 'local vector' is a species of limited distribution that has sufficient vectorial capacity to maintain localized endemicity without the main vector. In coastal regions of Africa, *Anopheles melas* and *Anopheles merus*, which develop in saltwater habitats, can be local vectors of *P. falciparum* where they have sufficient contact with humans. (iii) A 'bridge vector' is a species that can transmit an arbovirus from an amplifying host to a dead-end host. It is important, where possible, to assess putative vectors quantitatively by measuring their vectorial capacity, i.e. the average number of potentially infective bites that will ultimately be delivered by all the vectors that feed upon a single infective host in one day (Glossary, Appendix 3).

Mosquito species that are considered main vectors of infectious agents should satisfy the following criteria. (i) The mosquito and vertebrate hosts of the infectious agent occur sympatrically. They dwell in the same habitat at the same season, and in some cases occupy the same microhabitat. (ii) The mosquitoes feed preferentially on one or more species of amplifying host. (iii) The infectious agent has been isolated from wild mosquitoes. (iv) The mosquito population attains a sufficient size

relative to that of one or more amplifying host species. (v) In the laboratory, the mosquitoes are readily susceptible to infection by the infectious agent, do not block its full development and transmit it efficiently. (vi) The lifespan of a sufficiently high proportion of wild females exceeds the extrinsic incubation period of the infectious agent.

For a mosquito species to be confirmed as a main vector of an infectious agent in a given area it should, ideally, show the six characteristics listed above, but it is difficult to measure all of them. In practice, a putative vector is expected to show characteristics (iii) and (v) at least. The ability of a mosquito species to transmit an infectious agent, under experimental conditions, from infective to uninfected hosts does not, alone, establish that it is a natural vector. For example, some mosquitoes that can transmit yellow fever virus or certain *Plasmodium* species from host to host in the laboratory (Bates, 1949; Huff, 1965) have no association with those infectious agents in nature.

41.2 MODES OF TRANSMISSION

'Transmission' is a generic term used for the processes by which infectious agents, whether viruses or cellular organisms, symbionts or parasites, pass from one host to another. Transmission occurs in a variety of ways, which may be denoted by contrasting pairs of terms: direct or indirect transmission; mechanical or biological transmission; horizontal or vertical transmission.

When transmission is **direct**, the transmission stages of an infectious agent pass from one host individual to another of the same species (without the need for replication in another species). In the case of infectious agents that necessarily infect more than one species of host during the life cycle, **indirect transmission** occurs when the transmission stages of the infectious agent pass from one host species to a different host species (replication occurring in one of the species). Different modes of indirect transmission are described in the following sections.

41.2.1 Mechanical and biological transmission

When infectious agents are transferred from one vertebrate host to another by haematophagous arthropods, the mode may be mechanical or biological. In **mechanical transmission**, infectious agents are transferred from infected to uninfected vertebrate hosts on the mouthparts of haematophagous arthropods. A blood meal on an infected host must be followed immediately or within a few hours by a second blood meal on an uninfected host. In such cases, the infectious agents do not invade or reproduce within the arthropods, which cannot be described as hosts but are mechanical vectors. The best-known examples of mosquitoes serving as mechanical vectors are in the transmission of certain pox viruses, notably myxoma virus (Section 43.3). Mechanical transmission is a form of direct transmission, because the infectious organism does not need to replicate in the arthropod vector (in this example, in the mosquito).

Infectious agents that of necessity have two hosts in the life cycle, one a haematophagous arthropod and the other a vertebrate, pass between these hosts by **biological transmission**. The haematophagous arthropods are both hosts and vectors. Infectious agents that are ingested when the arthropod feeds on an infective vertebrate invade and reproduce within the arthropod host; when the arthropod feeds again later, the infectious agents pass to and invade the vertebrate. Infectious agents that have invaded a competent arthropod host must be able to survive in it, traverse a variety of barriers and reproduce. Most often they invade the salivary glands of the arthropod, and are carried into a new vertebrate host in saliva during a blood meal. For a vector population, the mean time between ingestion of an infected blood meal and attainment of competence to transmit the parasite by bite is the 'extrinsic incubation period'. Mosquitoes are the definitive hosts (Section 41.1.2) and vectors of many arboviruses, certain apicomplexans and certain filarioid worms. For a very few arboviruses (defined in the Glossary, Appendix 3), mechanical

transmission by mosquitoes supplements biological transmission (Section 44.4.2).

41.2.2 Horizontal transmission

The term horizontal transmission concerns transference of an infectious agent between any two individuals that are not related as parent and offspring. It embraces all modes of biological transmission other than vertical transmission (direct transference from a parent to its progeny). Horizontal transmission can occur between hosts of the same generation or from hosts of one generation to those of the next. It can involve transmission between hosts of the same species and between hosts of different species.

Horizontal transmission of mosquito parasites and pathogens between their hosts may occur in any of five different ways. (i) Ingestion by mosquito larvae of viral particles or microsporidial spores released on the death of infected hosts. (ii) Invasion by the motile stages of a parasite that are released on the death of infected hosts, e.g. theronts of *Lambornella*. (iii) Uptake of arbovirus or *Plasmodium* by mosquitoes feeding on infective vertebrate hosts. (iv) Transference of arbovirus or *Plasmodium* from infective mosquitoes to vertebrate hosts during blood feeding. (v) Transference of arbovirus in semen from infected male mosquitoes to uninfected females during copulation, so-called venereal transmission (Section 44.5).

41.2.3 Vertical transmission

(a) Biology

Vertical transmission is the direct transference of an infectious agent from one generation of its host to the next, i.e. from a parent organism to his or her progeny. The term 'vertical transmission' was coined to describe the mode of transference of a virus (*Mouse mammary tumor virus, Betaretrovirus*, family *Retroviridae*) from infected female mice to their sucklings in milk (Gross, 1949). Later, vertical transmission was shown to occur widely in the animal and plant kingdoms, and in many different forms (Fine, 1974, 1975). Most often, vertical transmission involves the passage of an infectious agent from an adult female host to her progeny, but a few infectious organisms can also be transferred in gametes from adult male hosts to their progeny, e.g. sigma virus in *Drosophila* (Section 41.3.2.b). Infectious agents that are perpetuated by vertical transmission alone satisfy one or more of a number of conditions: (i) they are transmitted to the progeny of both their male and female hosts; and (ii) if transmitted by female hosts alone, either the filial infection rate is consistently 100% or infected hosts have a selective advantage over uninfected hosts. The last situation is found in *Wolbachia*, in which infected ♀ × aposymbiotic ♂ crosses are fertile, whereas infected ♂ × aposymbiotic ♀ crosses are sterile (Section 46.9.4.b, Figure 46.21).

Theoretical analyses of virulence suggest that, when transmission of a parasite is vertical, selective forces will act to reduce virulence. A parasite that is transmitted largely or exclusively vertically should not harm its host, because the number of new infections depends upon the fecundity and fertility of the host (Sabelis and Metz, 2002). This is consistent with many empirical observations.

(b) Definitions of terms

Terms relevant to different aspects of vertical transmission were defined by Gross (1949), Burgdorfer and Varma (1967), Fine (1975) and Turell (1988). They are scattered through the Glossary (Appendix 3), but are brought together here. Definitions are always open to refinement, and some changes have been made for this volume.

Vertical transmission – transference of an infectious agent from a parent organism to his or her progeny.

Transovarian transmission – a means of vertical transmission in which infectious agents that have invaded oocytes when within the ovary are transmitted to a female's progeny.

Transovum transmission – a postulated means of vertical transmission in which, during ovulation, infectious agents invade chorionated ova (oocytes) and are transmitted to a female's progeny.
Trans-stadial transmission – passage of an infectious organism from one developmental stage of a host individual to a later stage.
Maternal infection rate – the proportion of a cohort or population of adult females that is infected at emergence, the females having infected germ cells.
Filial infection rate – the proportion of the progeny of an infected female that is infected through vertical transmission. (The developmental stage of the progeny is not specified.)
Vertical-transmission rate – the proportion of the progeny from a cohort or population of infected females that is infected through vertical transmission.
Effective vertical-transmission rate – the proportion of the progeny from a cohort or population of females, both infected and uninfected, that is infected through vertical transmission. (Proposed new term.)
Paternal vertical-transmission rate – of a cohort or population of infected males that had mated with uninfected females, the proportion of their progeny that is infected through vertical transmission.
Maternal vertical-transmission rate – of a cohort or population of infected females that had mated with uninfected males, the proportion of their progeny that is infected through vertical transmission.
Stable infection – an infection in which virtually all primordial germ cells of the host are infected so that all or virtually all gametes derived from them are infected. A female with a stable infection transmits the infectious agent to essentially all of her progeny.
Non-stable infection – an infection in which no primordial germ cells of the host are infected and in which relatively few or no germ cells become infected late in or after the completion of gametogenesis. A female with a non-stable infection transmits the infectious agent to only a proportion or to none of her progeny.

COMMENTS

(i) The adjective 'ovarian' is standard in medical dictionaries; 'ovarial' is a much older form (*Shorter Oxford English Dictionary*, 6th edn). Thus, 'transovarian transmission' replaces 'transovarial transmission'.
(ii) The term 'maternal infection rate' replaces the terms 'transovarial infection rate' and 'transovarial transmission rate', being more appropriate.
(iii) The term 'filial infection rate' concerns the progeny of an individual female. It has sometimes been used incorrectly, e.g. when the mean value for a number of filial infection rates was cited. The mean of a number of filial infection rates approximates the vertical-transmission rate.
(iv) In the definitions of 'filial infection rate' and 'vertical-transmission rate' the developmental stage of the progeny is not specified.
(v) For mosquitoes, the term 'vertical-transmission rate' is limited to transference of an infectious agent through the female line. It does not include possible venereal transmission after transference through the male line.
(vi) The terms 'paternal vertical-transmission rate' and 'maternal vertical-transmission rate' are not in common use; they are included here because of their use in certain simulation models (Section 41.4).
(vii) The quantitative terms defined above are not rates but proportions (see Glossary, Appendix 3, for both these words).
(viii) An oocyte (or ovum) is a female gamete, in which meiosis occurs. Very soon after an oocyte has been laid, female meiosis is complete, at which stage the oocyte becomes an egg.

(c) Occurrence in mosquitoes

Among the viruses and microorganisms that infect mosquitoes, two variants of vertical transmission have been described. (i) Transmission cycles are maintained by vertical transmission only. Infections of the rickettsial mutualist *Wolbachia* in mosquitoes are maintained in this way (Section 46.9). (ii) Transmission cycles involve both vertical and

horizontal transmission, notably La Crosse virus (Section 45.5.6), and for some microspordia. The situation in the few insect-only flaviviruses known to infect mosquitoes is variable (Section 43.2.2).

Three means of vertical transmission through the female line have been postulated. (i) *Transovarian transmission*: infectious agents invade female gametes, either by entering primordial germ cells or by entering oocytes before they have become enclosed by chorions; they survive through the egg stage and undergo trans-stadial transmission to the F_1 adult host. (ii) *Transovum transmission*: infectious agents invade chorionated oocytes during ovulation and while they are within the female genital ducts (possibly entering through the micropyle); they survive through the egg stage and undergo trans-stadial transmission to the F_1 adult host (Section 44.6.1.b). (iii) *Contamination of the oocyte surface*: during ovulation and while the oocytes are within the female genital ducts, infectious agents adhere to the chorion and remain attached throughout oviposition. Later, they are ingested by and infect newly hatched larvae. No evidence in support of this hypothesis has been produced, and it is not considered further.

When vertical transmission occurs, the infectious agents are equally distributed between male and female progeny. In *Drosophila melanogaster* infected with sigma virus (which is not an arbovirus), vertical transmission to the following generation is possible through both female and male lines (Section 41.3.2.b). With mosquito-borne arboviruses, only vertical transmission through the female line is known. Infection of spermatozoa and direct transference from adult male mosquitoes to their progeny has never been described, but it has been looked for in only one or two species, so a blanket dismissal of the possibility would be rash. Some arboviruses can be transmitted horizontally by venereal transmission, i.e. from male to female mosquitoes during copulation, and this can lead to indirect transference of arbovirus from adult male hosts to their progeny.

Infectious agents can be perpetuated in the long term by vertical transmission alone, but only if certain conditions are satisfied. The phenomenon has been approached in two ways. (i) Through the concept of 'stable infections', in which the infectious agent infects primordial germ cells and hence is present in all gametes (Section 41.3.2.b). (ii) Through simulation models in which the variables are the relative rates of fertility, viability and vertical transmission (Section 41.4.1). *Wolbachia* infections in mosquito populations are maintained by vertical transmission alone because virulence is low and also because, in situations in which infected and uninfected host populations occur sympatrically, they provide a selective advantage for infected over uninfected host populations (crosses between aposymbiotic females and infected males are infertile (Section 46.9)).

Transmission cycles that include phases of both horizontal and vertical transmission have been investigated in detail in two groups of infectious organisms: microsporidia and viruses. Among the microsporidian parasites of mosquitoes, the alternation of phases of vertical and horizontal transmission has two variants. (i) Transmission cycles comprise two phases of proliferation which are passed in different individuals of the same mosquito species. One phase starts with the infection of larvae by vertical transmission; growth and multiplication of the parasite within the host larvae lead to death of the larvae and the release of spores. The second phase, of horizontal transmission, starts with the ingestion of such spores by other larvae of the same species and results in non-fatal infection. This is known to occur for *Culicospora magna* infecting *Culex restuans* (Volume 4, Chapter 53). (ii) Transmission cycles comprise two proliferative phases, one of which is passed in a mosquito host and the other in a different organism. Species of *Amblyospora* and *Parathelohania* usually infect mosquitoes as the definitive host and copepods as the intermediate host. Most of the parasite species are transmitted vertically from adult female mosquitoes to their progeny via binucleate spores, then horizontally from the infected mosquito larvae to copepods via meiospores, and horizontally from copepods to mosquito larvae via uninucleate spores.

Coupling of vertical and horizontal transmission has been reported for viruses (non-arboviruses) of the families *Baculoviridae* and *Iridoviridae*

that infect culicid hosts: vertically infected larvae die, releasing virions that may be ingested by healthy larvae (Section 43.2.3.d). Horizontal transmission between mosquito and vertebrate hosts and viral amplification in the vertebrate hosts are important elements in the transmission cycles of mosquito-borne arboviruses. Vertical transmission in the mosquito host has been reported from the field for a small number of bunyaviruses, and may be important for their perpetuation (Section 44.6.2). The evidence for stable infections of arboviruses in mosquitoes is slender (Section 44.6.4), and it is thought that most such infections are non-stable – in which case the vertical transmission rate is <1.0 and the prevalence rate of infection declines from one host generation to the next. Therefore, perpetuation of the virus in the medium term depends upon its amplification during phases of horizontal transmission.

41.3 STABLE AND NON-STABLE INFECTIONS IN *DROSOPHILA*

41.3.1 Introduction

Stable infections are those in which virtually all primordial germ cells of the host are infected, so that all or virtually all gametes derived from them are infected. A female with a stable infection transmits the infectious agent to virtually all her progeny. In non-stable infections, no primordial germ cells of the host are infected and germ cells become infected late in gametogenesis or after its completion. A female with a non-stable infection transmits the infectious agent to a proportion of her progeny. These two types of infection have been characterized most fully with infections of sigma virus in *Drosophila*, and it is from comparisons with those characteristics that claims for stable infections in mosquitoes should be assessed. Note that the 'stability of a virus', measured by the loss of infectivity when outside a host, is a different concept and is not considered here.

Our knowledge of stable infections derives principally from studies of infections of *Drosophila melanogaster* with sigma virus, a subject that is reviewed in detail in the following sections. Sigma *virus* (SIGMAV) was classified with the family *Rhabdoviridae* but was not assigned to any genus (Fauquet, 2005). Later, closely related viruses were isolated from *D. obscura* and *D. affinis*. Genomic sequencing and phylogenetic analysis showed the three species forming a deep-branching clade in the *Rhabdoviridae* tree that merited recognition as a new genus. All three species are transmitted vertically, in males through sperm and in females through oocytes (Longdon *et al.*, 2010, 2011). Host/virus interactions have been investigated in colonized and wild populations of *D. melanogaster* and sigma virus in France, and the concept of stable and non-stable infections arose from those studies.

Whether or not infections with yellow fever virus in certain of its mosquito hosts are stable or unstable could be important for explanations of the ability of the virus to survive through long, dry seasons. Evidence for stable infections of arboviruses in mosquitoes is discussed in Section 44.6.4. Infections of mosquitoes by *Wolbachia* have the characteristics of stable infections (Section 46.9).

41.3.2 Laboratory investigations of sigma virus in *Drosophila*

(a) Hypersensitivity to carbon dioxide

Flies of certain strains of *Drosophila melanogaster* when placed in an atmosphere rich in CO_2 become inert within a few seconds but recover entirely on return to a normal atmosphere, even after 5 h exposure. The flies of some other strains fail to recover after even a short exposure, especially at lower temperatures. This phenomenon was first observed by L'Héritier and Teissier (1937), who reported that flies of a strain carrying the gene *ebony* were 'sensible' (sensitive) to CO_2, dying upon exposure, whereas other strains were unaffected, or insensitive. The sensitivity trait was found to be inherited, but independently of the chromosomes; it was ascribed to the presence in the cytoplasm of a factor named σ (L'Héritier and Teissier, 1938a,b). The susceptibility to CO_2 that leads to death was never transferred from sensitive to insensitive strains by contact, but could be

transferred by the injection of haemolymph, or by the transplantation of ovaries or cerebral ganglia from sensitive into insensitive flies of either sex. Some progeny of the recipient females, but not of recipient males, were sensitive. L'Héritier and de Scoeux (1947) postulated that sensitivity was due to a 'virus like cytoplasmic hereditary unit'. Later, this agent was shown to be a virus that multiplies in host cytoplasm; this was named sigma virus. The action of CO_2 on the flies is chemically specific; no other gas produces comparable effects. In a test developed by Plus (1954), flies were exposed to pure CO_2 at 13 °C for 15 min, and any fly which could not right itself and walk after 15 min in an atmosphere of pure air was classed as sensitive.

Later, the infectious agent was shown to be a virus that multiplies in host cytoplasm, and it was named sigma virus. The action of CO_2 is specific to that compound; no other gas produces comparable effects. The effects of CO_2 on virus-infected flies are the result of its actions within the thoracic ganglia. Sensitivity allows easy separation of uninfected flies, which quickly recover from brief exposure (L'Héritier, 1970; Brun and Plus, 1980).

In North America, sampling revealed moderate rates of CO_2 sensitivity in wild-caught flies of *Drosophila affinis* (18–39% in small samples from 17 populations) and *Drosophila athabasca* (3–20% in six populations). A large sample of *D. melanogaster* from Nebraska showed 1.6% sensitivity. Lines that were 100% sensitive were developed from single sensitive females of *D. affinis* and *D. athabasca* (Williamson, 1961). The causal agent, which was heritable and transmissible by inoculation, was taken to be sigma virus (Brun and Plus, 1980).

(b) Stable and non-stable infections

Sigma virus is perpetuated solely by vertical transmission; infection by horizontal transmission has never been reported and no possible mechanism for the natural horizontal transmission of the virus is known. In populations of *D. melanogaster* in which the virus is endemic, perpetuation depends on the efficiency of its transmission from adults to offspring. Low efficiency transmission was regarded as equivalent to the classical condition of virus multiplication within host cells and classed as a 'non-stabilized' state. High efficiency transmission was taken to be a veritable integration of the virus with the host cells, and was classed as 'stabilized' (Brun and Sigot, 1955; Brun and Plus, 1980). Those terms are still widely used, as in 'stabilized females', 'stabilized males' and 'stabilized line'. They will be replaced in this volume by the terms 'stable infection' and 'non-stable infection', which are thought to be more appropriate.

In flies with **stable infections**, virtually all primordial germ cells are infected with sigma virus, but by chance a proportion of 0.01 to 0.001 remain free from virus particles. Consequently, within the ovaries of adult females, virtually all of the primordial germ cells and oogonia are infected, and all oocytes produced by those ovaries are infected. Therefore, a female with a stable infection transmits the virus to virtually all her progeny. Early embryos are rich in virions, and their pole cells (the germ-line cells) are infected from the outset. If such an embryo develops to an adult female, all its oocytes will have been infected very early and the stable infection will be perpetuated. In *D. melanogaster*, once initiated, the stable infection with sigma virus will be maintained for generations through the maternal lineage (Figure 41.1). In contrast, in *D. affinis* and *D. athabasca*, CO_2 sensitivity is not only maternally inherited but is inherited by males, which have inherited their sensitivity only from their fathers (Williamson, 1961).

If the embryo develops to an adult male, the infection will be stable, but only some of its spermatozoa will transmit the virus. When a male with a stable infection inseminates a non-infected O/O female (see below), none of the progeny will have stable infections, but some will have non-stable infections. The term 'valence' (English, valency), when used with reference to the parental males in such crosses, is quantified as the frequency of infected flies among their progeny.

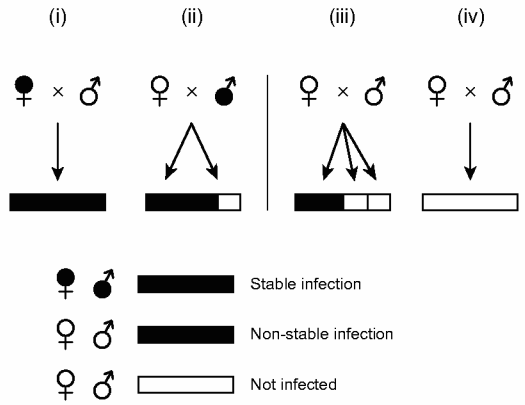

Figure 41.1 Diagram illustrating the transmission of sigma virus to the progeny of *Drosophila melanogaster* when infected females have mated with uninfected males, and when infected males have mated with uninfected *O*/*O* (permissive allele) females. The outcomes are further affected by whether the infections are stable or non-stable. (After Fleuriet, 1988.)
Left. The parents of one or other sex have stable infections.
(i) The female parents have stable infections, and the maternal vertical-transmission rate is 1. Outcome: all progeny have stable infections.
(ii) The male parents have stable infections, and the paternal vertical-transmission rate (valency) is variable. Outcome: any infections are non-stable.
Right. The parents of one or other sex have non-stable infections.
(iii) The female parents have non-stable infections. Outcome: the maternal vertical-transmission rate is variable; some of the progeny develop stable infections (neostabilization), others develop unstable infections and the rest are uninfected.
(iv) The male parents have non-stable infections. Outcome: the paternal vertical-transmission rate is 0.

When infected males from French populations were mated with uninfected females of the same origin, the F_1 valencies ranged from 0 to 1.

In flies with **non-stable infections**, virions are disseminated throughout the adult body, and the amount of virus is higher than in flies with stable infections. However, the embryo contains few viral genomes, and the primordial germ cells are not infected from the outset. In females, a few oocytes may become infected in one or other of two ways: either during late oogenesis by some of the virus that is disseminated throughout the body, or after oogenesis and during fertilization by virus present in infected sperm. Embryos that develop from such oocytes are not rich in viral genomes, and in only a proportion of them do the germ-line cells become infected. Three types of adult female progeny can be distinguished: (i) females that are uninfected; (ii) females whose oocytes become infected late in oogenesis and which acquire non-stable infections; (iii) females whose oocytes become infected early in oogenesis and which acquire stable infections (a process termed neo-stabilization) (Figure 41.1). When embryos that are poor in viral genomes give rise to adult males, the spermatocytes do not become infected during spermatogenesis, and the males do not transmit the virus to their offspring (Bregliano, 1970; Brun and Plus, 1980; Fleuriet, 1982a, 1988).

(c) *Genetics in* Drosophila

In *D. melanogaster*, the gene *ref(2)P* is polymorphic and its several alleles can be classified as permissive or restrictive. Two alleles, $ref(2)P^O$ and $ref(2)P^P$ (sometimes designated O and *P*), have been extensively studied. The presence of the *P* allele causes interference with the multiplication of sigma virus in the fly. The permissive and restrictive alleles are codominant, and their products compete with one another to permit or restrict multiplication of sigma virus. In heterozygotes, the presence of a permissive allele counteracts the activity of a restrictive one. All natural populations of *D. melanogaster* examined were polymorphic for these alleles, *P* being in the minority (Fleuriet, 1982b, 1988; Wyers *et al.*, 1995).

Genes of both the virus and its host contribute to the maintenance of sigma virus in *Drosophila*. Two types of sigma virus coexist in nature. Type I virus survives in flies carrying permissive (*O*) alleles, but is very sensitive to the restrictive (*P*) alleles. Type II virus is more resistant to the *P* alleles, but a wide range of sensitivities to *P* is found among Type II clones. In a fly population perpetuating both P^O and P^P alleles, Type I virus cannot persist whereas Type II can be maintained and can spread (Fleuriet, 1982b).

41.3.3 Field studies of sigma virus in *Drosophila*

The prevalence rate of sigma virus in populations of *D. melanogaster* can vary widely, both geographically and in time, reflecting interactions between virus and host. In northern and central France during the 1970s, the prevalence rate was about 0.15–0.20. It was lower in some other parts of the world. In contrast, when experimental populations were reared in cages, the virus was able to infect almost all of the flies (Fleuriet, 1982b, 1988). Analyses of 38 natural populations of *D. melanogaster* in the Languedoc region of southern France between 1983 and 1991 revealed a year-by-year increase in the prevalence of infected flies, followed by a progressive decrease. Between 1983 and 1988 the mean prevalence rate of infection rose from 0.15 to 0.65; between 1989 and 1991 it fell from 0.65 to 0.30. In all French populations the efficiency of transmission by males decreased. These changes in prevalence were associated with changes in both virus and host. (i) Adaptation of the virus – between 1983 and 1991, the prevalence rate of Type I virus, which is sensitive to the *P* allele, declined from 0.47 to 0.03, while that of Type II virus increased from 0.53 to 0.97. The effect of this change was enhanced by a decrease in the sensitivity of the Type II virus to the flies' restrictive allele (*P*). (ii) Adaptation of the flies – the occurrence of the restrictive allele (*P*) increased slightly but progressively in fly populations from 1984 to 1991 (Fleuriet, 1990; Fleuriet *et al.*, 1990; Fleuriet and Periquet, 1993).

Flies that had been collected from wild French populations, and that were infected or not infected with sigma virus, were compared for physiological traits that included viability of the developmental stages, male and female fertility, female longevity and sexual selection. Infection modified the flies' fitness only slightly; the only significant difference that was found was a lower viability, in some replicates, of the developmental stages of the progeny of infected females (Fleuriet, 1981). Further experiments showed that, when females had mated with O/O males, the egg-to-adult viability of the progeny of infected females was significantly lower than that of the progeny of uninfected females. This viability was largely unaffected when infected females had mated with O/*P* males (Fleuriet, 1994). In contrast to these results with initially wild-caught flies, laboratory clones of sigma virus often were harmful to their hosts (Fleuriet, 1988).

Transmission through male as well as female hosts can make possible the survival of an infectious agent from generation to generation by vertical infection alone (Section 41.4.1). For that reason, neostabilization is essential for the perpetuation of sigma virus infections in wild populations of *D. melanogaster*. Virtually all the progeny of females with stable infections of sigma virus will carry stable infections, but the progeny of crosses between uninfected females and males with stable infections either have non-stable infections or are uninfected. However, neostabilization of infections occurs among the progeny of the F_1 daughters that had acquired non-stable infections (Figure 41.1). For 12 French populations, neostabilization values of 0.92–0.97 were found; the mean value in France was about 0.75 (Fleuriet, 1982a, 1988).

41.4 SIMULATION MODELS OF TRANSMISSION CYCLES

41.4.1 Modelling cycles that are maintained by vertical transmission alone

Fine (1975) developed a general model for transmission cycles of infectious organisms maintained through successive generations of their host populations by vertical transmission alone. The model assumes that an infection is present in a proportion of the adult members of a sexually reproducing population. It assumes also that (i) infection persists throughout the lifetime of vertically infected individuals, (ii) infection has similar effects on males and females of the host species, and (iii) mating occurs at random in the host population. Development of the model led to

a 'fundamental vertical transmission equation' (Eqn 41.1, shown in the caption to Figure 41.2), which indicates the quantitative contribution of vertical transmission to the prevalence rates (see Glossary) of infection in subsequent generations.

As noted in Figure 41.2, given any set of values for the five variables listed in the caption, the solution of the fundamental vertical transmission equation can readily be obtained. For a vector population *B*, by repeatedly substituting the solution B_a' (the prevalence rate of inherited infection among adult members of the progeny generation) for B_a (the prevalence rate – or proportion – of infection among adult ovipositing vectors) in the right-hand side of the equation, one can iterate the calculation and so determine the prevalence rates of infection that would be found in successive generations of hosts. The results of two such iterations are illustrated in Figure 41.2; both have the initial prevalence rate set at 0.5. Under one set of variables (solid circles) the prevalence rate increases and would stabilize at approximately 0.83, compatible with infection being perpetuated by vertical transmission alone. Under the second set of variables (hollow circles) the prevalence rate declines steadily, ultimately to zero, so infection cannot be maintained by vertical transmission alone. This latter condition is typical of transmission cycles in

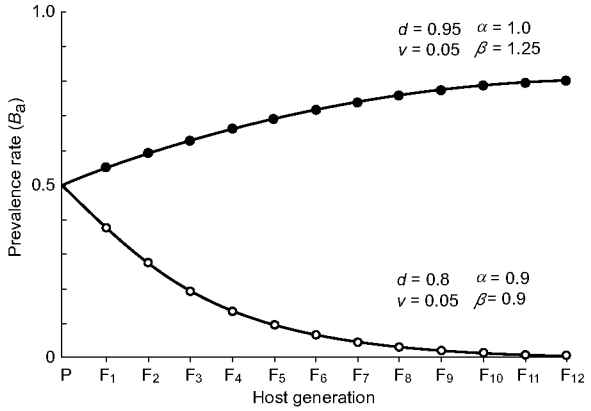

Figure 41.2 Calculated changes in the prevalence rates of an infection when maintained through 12 generations of its hosts by vertical transmission alone. The prevalence rate in the parental generation was set at 0.5, and two sets of variables were applied. The curves were obtained by iteration of the 'fundamental vertical transmission equation' (Eqn 41.1; see below). (Modified from Fine, 1975.) Each point on the curves is a prevalence rate (Ba) for the generation specified on the horizontal axis.

The 'fundamental vertical transmission equation' (41.1) provides an initial measure of the quantitative contribution of vertical transmission to the prevalence rates of an infection through successive generations of the host:

$$B_a = \frac{\beta[B_a\alpha d(1-B_a+B_a\alpha)+B_a\alpha v(1-B_a+B_a\alpha-B_a\alpha d)]}{\beta[B_a\alpha d(1-B_a+B_a\alpha)+B_a\alpha v(1-B_a+B_a\alpha-B_a\alpha d)]+(1-B_a+B_a\alpha-B_a\alpha d)(1-B_a+B_a\alpha-B_a\alpha v)} \quad 41.1$$

where

B_a' is the prevalence rate of inherited infection among adults of the progeny generation;

B_a is the proportion (or prevalence rate) of the adult ovipositing members of the vector population (*B*) that are infected;

d is the maternal vertical-transmission rate, i.e. the prevalence rate of infection among the progeny of infected females when mated with uninfected males (symbol r in Fine, 1975);

v is the paternal vertical-transmission rate, i.e. the prevalence rate of infection among the progeny of infected males when mated with uninfected females;

α is the relative fertility (number of progeny) of infected adults compared with their uninfected peers;

β is the survival rate (to reproductive age) of vertically infected individuals compared with uninfected individuals.

Given any set of values for B_a, d, v, α and β, the solution of the fundamental equation is straightforward. By repeatedly substituting the solution B_a' for B_a in the right-hand side of the equation, one can iterate the calculation, determining the prevalence rates of infection that would be found in successive generations of hosts, as long as the assumptions hold. The results of two such iterations are illustrated in this figure.

which infectious agents infect both vertebrate hosts and arthropod vectors, and that include phases of both horizontal and vertical transmission.

There is a simpler method of predicting whether an infectious agent can be maintained in successive generations of its host by vertical transmission alone. Where

$$\alpha\beta(d+v)>1 \tag{41.2}$$

the conditions are sufficient for survival from generation to generation solely by vertical transmission. In this expression, a is the relative fertility of infected compared with uninfected adults, β is the relative viability of infected compared with uninfected individuals, d is the maternal vertical-transmission rate and v is the paternal vertical-transmission rate (see Table 41.2 for fuller definitions). In cases where the expression

$$\alpha\beta(d+v)<1 \tag{41.3}$$

applies, the prevalence rate would decline steadily, and ultimately to zero, in the absence of amplification during phases of horizontal transmission (Fine, 1975). Fleuriet (1988) pointed out that there are several possible situations, all assuming that infection does not greatly affect host fitness, i.e. $a\beta \cong 1$.

(1) Vertical transmission occurs through both female and male gametes.

(1a) If $d + v > 1$, then the infectious agent persists in the population. It is transmitted through both female and male gametes, and if the combined maternal and paternal vertical-transmission rates are sufficient the agent may be maintained in a population by vertical transmission alone, even if infection reduces host fitness ($a < 1$, $\beta < 1$), provided that $a\beta(d + v) > 1$. That was the case with sigma virus in *Drosophila melanogaster* in France, where infections persisted at different prevalence rates in different host populations (Section 41.3.3).

(1b) If $d + v < 1$, then the infectious agent is eliminated.

(2) Vertical transmission is through female gametes only.

(2a) If $d = 1$, then infected females transmit the agent to all their progeny. Many microorganisms are thought to be maintained in this manner, notably bacterial mutualist symbionts of arthropods, which are so intimately associated with their hosts that they never need to escape the confines of the host organism. Unless the infectious agent confers some advantage on its host, it is likely that, because of drift, the agent will eventually be eliminated. Where *Drosophila simulans* contains the symbiont *Wolbachia*, infected host populations gain advantage over aposymbiotic populations because crosses between aposymbiotic females and infected males are infertile (Section 46.9.6.c).

(2b) If $d < 1$, then the prevalence rate of infection steadily decreases towards zero.

Whether venereal transmission, which has been demonstrated experimentally in mosquitoes (Section 44.5), can enhance significantly the maintenance of an infectious agent by vertical transmission can be tested by use of a simulation model (Eqns 44.7, 44.8). Assuming an efficiency of venereal transmission of 0.75, and a daily survival rate of 0.90, the model indicates that, when vertical-transmission rates at emergence range from 0.002 to 0.08, venereal transmission increases the summed contribution of vertically infected females to egg deposition in the first and second gonotrophic cycles by 0.24–0.26 (24–26%) (Table 44.5). At much higher vertical-transmission rates, the extent of this contribution by venereally infected females is greatly reduced. Thus, when the vertical-transmission rate at emergence is 0.95, which approaches the rate of 1.0 needed for perpetuation solely by vertical transmission, venereal transmission would increase vertical transmission to the next generation by only 0.013 (1.3%).

41.4.2 Modelling cycles that involve both vertical and horizontal transmission

Fine and LeDuc (1978) developed quantitative models of the natural transmission cycle of Keystone virus, a cycle that involves *Ochlerotatus atlanticus* as mosquito host and vector, and certain

small mammals as vertebrate hosts. The filial infection rate among the progeny of infected female *Oc. atlanticus* was taken to be <1.0 (characteristic of non-stable infections), therefore the vertical-transmission rate was <1.0 and amplification of virus during phases of horizontal transmission was essential for its perpetuation. More recently, some authors have described certain infections of mosquitoes by an arbovirus as stable. If their claim is confirmed, additional simulation models will be needed.

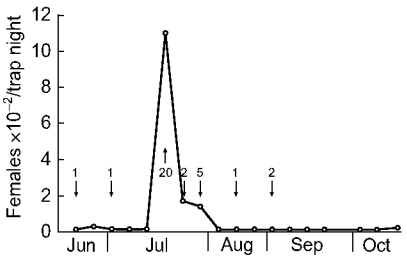

Figure 41.3 Relative numbers of adult female *Ochlerotatus atlanticus* at Pocomoke Cypress Swamp, Maryland, during 1971, based on light-trap samples. (After LeDuc *et al.*, 1975c.) The arrows show the dates of isolation of Keystone virus from those mosquitoes, while the associated numerals show the numbers of isolates. As in the original figure, the datum points are spaced evenly along the *x*-axis although the intervals between sampling varied; consequently, the apparent durations of the monthly periods are irregular.

(a) Ecology of Keystone virus and its hosts

Keystone virus (KEYV), a serotype of the California antigenic group (*Orthobunyavirus*, family *Bunyaviridae*), is maintained over a wide area of the south-eastern USA in a sylvatic cycle that involves vertebrates as amplifying hosts and *Ochlerotatus atlanticus* as vector. The system has been investigated most thoroughly at the Pocomoke Cypress Swamp in Maryland (Figure 45.3), where the virus is endemic but near the northern limit of its range. Here, *Oc. atlanticus* usually has just one generation per year. This mosquito overwinters in the egg stage, the eggs having been laid in heavily shaded, shallow depressions in the ground. Hatching is triggered by the first major summer rain, which may occur at any time between June and September. In this area, a minimum rainfall of 3 inches (7.6 cm) in 24–48 h is needed to produce suitable temporary pools. Such rains may occur only once a summer, and in most years only a single brood emerges. The aquatic stages develop rapidly and the adults emerge almost simultaneously (Figure 41.3) (LeDuc *et al.*, 1975a,c).

Characteristically for a floodwater mosquito species, the flight period for *Oc. atlanticus* is short, lasting about a month during the summer, although small numbers of adults can be found earlier and later. The first isolation of KEYV occurs on virtually the first appearance of adults, when the females are unlikely to have taken a blood meal (Figure 41.3), so the vector population must be vertically infected at the time of emergence. The isolation of KEYV from wild-caught larvae of *Oc. atlanticus*, and from adult males and females reared from wild-caught larvae, confirmed that vertical transmission occurs in nature (LeDuc *et al.*, 1975c).

Ochlerotatus atlanticus is known to be an opportunistic feeder which will attack mammals, reptiles and birds, and this was confirmed at the Pocomoke Cypress Swamp (LeDuc *et al.*, 1972). Among the range of its wild mammalian hosts, rabbits and grey squirrels became viraemic after experimental infection (Table 41.1). Studies using rabbits as sentinels suggested that a high proportion of the susceptible mammalian hosts became infected with KEYV when vertically infected females first fed, and developed a viraemia that lasted 2–4 days. Surviving females were said to blood feed repeatedly. Some mosquitoes taking second or later blood meals became infected by feeding on viraemic hosts, and in that way horizontal transmission of the virus led to an increase in the prevalence rate of infection in the vector population. During the summer months, between 11 June and 8 September 1971, the overall MIR (minimum infection rate)/1000 of females captured in light traps was 4.0 (n = 7964) (LeDuc *et al.*, 1975a,b; Fine and LeDuc, 1978). In each gonotrophic cycle, females laid eggs determined for winter dormancy, and some of the eggs

Table 41.1 Interactions between *Ochlerotatus atlanticus*, Keystone virus and mammalian hosts in Pocomoke Cypress Swamp, Maryland. (From LeDuc *et al.*, 1975c.)

Host	*Engorged C. atlanticus** (%)	*Seropositive for KEYV* (%)	*n*	*Viraemia after experimental infection*
Whitetail deer	34	9.9	121	– †
Rabbit (domesticated)	12	33.3	3	+
Grey squirrel	6	29.6	27	+ ‡
Racoon	4	17.9	39	+ (low)
Opossum	2	0	18	–
Turtles	15	0	227	–
Others	27	–	–	–

*, Total of 436 blood meals.
†, A few wild whitetail deer had detectable neutralizing antibody to KEYV.
‡, Up to 10^5 PFU ml^{-1}.
– indicates test not done.

deposited by infected females contained virus. Because not all eggs laid by infected females contained virus, the filial infection rate among dormant eggs was lower than the maternal infection rate (Fine and LeDuc, 1978).

(b) *A simplistic model*

In developing a simulation model of the transmission cycle of Keystone virus, Fine and LeDuc (1978) first formulated a 'purposefully over simplistic' model that involved five variables (α, β, v, d, B_a), which are defined in Table 41.2. It was assumed that infection with KEYV has no selective effect on its vector, *Oc. atlanticus*, and that all infections are non-stable. We note that B_a is the prevalence rate of infection among ovipositing females, regardless of age, and that d is the maternal vertical-transmission rate among the progeny of uninfected ♀♀ × infected ♂♂. It follows that the prevalence rate of vertically transmitted infections among the progeny of each generation is equivalent to $B_a \times d$, and that at the end of any season B_ad will be the prevalence rate among both the overwintering eggs and the newly emerged adults that have developed from them. Thus, the model specifies the proportion of mosquitoes that are potentially infective to vertebrates at their first blood meal, i.e. before any horizontal transmission (Figure 41.4). Because $d < 1$, then $B_ad < B_a$. The predicted difference in prevalence rate of infection between parental mosquitoes and their progeny, $(B_a - B_ad)$, must be compensated for by amplification during horizontal transmission in each mosquito generation.

(c) *An advanced model*

A more detailed and advanced simulation model of Keystone virus transmission was also formulated by Fine and LeDuc (1978). Definitions of the symbols used are given in Table 41.2. The model comprised two phases.

Phase (i). Transmission to amplifying hosts during the vectors' first gonotrophic cycle (see Glossary, Appendix 3). The expression B_ad described the prevalence rate of infection among newly emerged mosquitoes; it specifies the proportion of mosquitoes which are potentially infective at their first blood meal and before any horizontal transmission. (The fundamental vertical-transmission equation (Eqn 41.1, in Figure 41.2) could also be used.) After females of *Oc. atlanticus* had taken their first blood meal,

Table 41.2 Symbols of variables used in models of transmission cycles of Keystone virus maintained by both horizontal and vertical transmission. (From Fine and LeDuc, 1978.)

α = relative fertility rate (infected relative to uninfected adults)
β = relative survival rate to reproductive age (of infected progeny relative to uninfected)
B_a = prevalence rate of viral infection among adult ovipositing females of a vector population B; equivalent to the proportion of all egg batches that are deposited by infected females if fertility is impaired)
$B_a d$ = prevalence rate of vertical infection among newly emerged mosquitoes (i.e. the initial prevalence rate)
$B_a di$ = probability that a mosquito taking its first blood meal is infectious
$1 - B_a di$ = probability that a mosquito taking its first blood meal is not infectious
$(1 - B_a di)^n$ = probability that none out of n mosquitoes, taking their first blood meals, is infectious
$1 - (1 - B_a di)^n$ = probability that at least one out of n mosquitoes, taking their first blood meals, is infectious
d = maternal vertical-transmission rate (prevalence rate of infection among the progeny of a cohort of infected females when mated with uninfected males)
f = proportion of mosquitoes that become infected by a single blood meal on a viraemic host
h_v = effective incidence rate of virus infections in the vertebrate population after the first mosquito blood meals
h_{m2} = incidence rate of virus infections in the mosquito population at the time of the second blood meal
i = proportion of infected mosquitoes that are infective to vertebrates
M = number of vectors that oviposit at least once
n = number of vectors per susceptible host at time of initial blood feeding
p = probability that a female mosquito survives through a gonotrophic cycle
S = proportion of all vector blood meals that are taken from susceptible host species
s = proportion of individuals of susceptible host species that lack immunity to virus at onset of transmission season
Ss = proportion of blood meals taken on susceptible and potentially viraemic hosts
π_1, π_2, π_3, etc. = proportion of females of vector species that are infected when taking their first, second, third, etc. blood meals
v = paternal vertical-transmission rate (prevalence rate of infection among the progeny of a cohort of infected males when mated with uninfected females

the effective incidence rate (h_v) of KEYV infections in the vertebrate population was expressed as

$$h_v = Ss\left\{1-(1-B_a di)^n\right\} \tag{41.4}$$

where S was the proportion of blood meals taken on susceptible host species; s was the proportion of individuals of susceptible host species that lacked acquired immunity at that time; the product Ss was the proportion of all blood meals taken on hosts able to both become infected and become sufficiently viraemic to transfer virus back to mosquitoes; i was the proportion of infected mosquitoes that were infective; and n was the number of vectors per susceptible vertebrate host at the time of feeding. The symbol h_v was derived from the phrase 'horizontal transmission to vertebrates'. The prevalence rate of infection among mosquitoes surviving to oviposit at the end of the first gonotrophic cycle was taken to be the prevalence rate of vertical infection ($B_a d$).

Phase (ii). Transmission from the amplifying hosts to infected and uninfected mosquitoes during the second gonotrophic cycle. The incidence rate of infection among mosquitoes that succeeded in taking a second blood meal (h_{m2}) can be equated with the incidence rate of viraemia in the vertebrate population, and the probability (f) that they will become infected; therefore

$$h_{m2} = fSs\left\{1-(1-B_a di)^n\right\} \tag{41.5}$$

The same incidence rate can be expressed differently as

$$h_{m2} = \frac{B_a(1-d)}{p(1-B_a d)} \tag{41.6}$$

where p is the probability of a female mosquito surviving through a gonotrophic cycle. Combin-

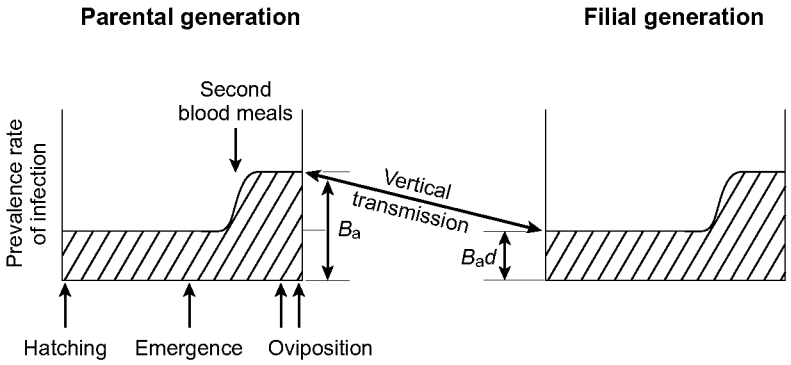

Figure 41.4 Diagram illustrating the relationship between the prevalence rates of Keystone virus infection in its mosquito host, *Ochlerotatus atlanticus*, and the developmental stages of two generations of that host. (After Fine and LeDuc, 1978.) Each box is constructed as an age × prevalence graph for a generation of vectors. The prevalence rate of infection ($B_a d$) is low throughout the aquatic stages, but increases when adult mosquitoes feed on viraemic vertebrates. The effective prevalence rate of infection among ovipositing females is then raised to B_a. Of the eggs laid by an infected female, only a proportion (d) carry virus, so the prevalence rate falls to $B_a d$ at the beginning of the next generation. The prevalence rate must be increased by the amount ($B_a - B_a d$) during each generation. No direct estimates of d had been made for KEYV in *Oc. atlanticus*, so the shape of the curves is speculative.

ation of a number of epidemiological elements led to the 'basic transmission model', which predicts the average number (n) of first blood meals that must be taken by the mosquito population on each susceptible vertebrate host to maintain the transmission cycle of Keystone virus in the Pocomoke Swamp. Thus

$$n = \frac{\log\left(\dfrac{pfSs(1-B_a d)-B_a(1-d)}{pfSs(1-B_a d)}\right)}{\log(1-B_a di)} \qquad (41.7)$$

The implications of this basic transmission model (Eqn 41.7) are illustrated in Figure 41.5, which shows the relationship between the number of first blood meals that need to be taken on each susceptible host (n) and the product '$pfSs$' at two different values of the maternal vertical-transmission rate (d). The extent to which the maternal vertical-transmission rate affects the number of first blood meals that must be taken on each vertebrate host for the transmission cycle to be maintained is evident. The product term '$pfSs$' is a measure of the proportion of the initial female mosquito population that both survives to take a second blood meal and becomes infected, and which, therefore, produces infected diapause eggs.

Two assumptions are inherent in the 'basic transmission model' (Eqn 41.7): (i) all vector-to-vertebrate transmission occurs at the first blood meal; and (ii) all vertebrate-to-vector transmission occurs at the second blood meal. Because, probably, these assumptions are not strictly true, an equation was developed describing the prevalence rate of infection (B_a) among ovipositing females in all gonotrophic cycles. Thus

$$B_a = \frac{M\pi_1 + Mp\pi_2 + Mp^2\pi_3 + Mp^3\pi_4 + \ldots}{M/(1-p)} \qquad (41.8)$$

where M is the number of vectors that oviposit at least once, and π_1, π_2, etc. are the proportion of females of vector species that are infected when taking their first, second, third, etc. blood meals. This simplifies to

$$B_a = (1-p)(\pi_1 + p\pi_2 + p^2\pi_3 + p^3\pi_4 + \ldots) \qquad (41.9)$$

Fine and LeDuc (1978) commented that mathematical models can be useful in highlighting the types of field data needed for evaluating virus transmission, and in assessing the validity of such data, and that, further, they provide a rigorous means of testing qualitative hypotheses about how complex systems function in nature. Their 'simplistic model' of KEYV transmission was designed

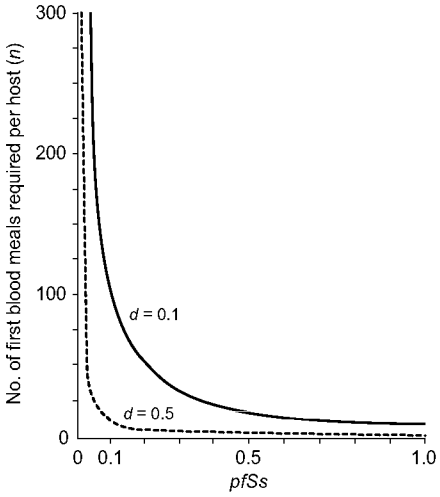

Figure 41.5 Plots of the average numbers (n) of first blood meals that need to be taken on each susceptible host in a vertebrate population to maintain the Keystone virus transmission cycle; shown at two different vertical transmission rates (d). Solid curve, d = 0.1; broken curve, d = 0.5. The graph represents solutions to Eqn 41.7, with the assumption that the prevalence rate of viral infection (B_a) among adult mosquitoes lies within the range $0.001 < B_a < 0.005$, as found in field isolations. In nature, the independent variable '$pfSs$' (see text) is probably <0.1. (From Fine and LeDuc, 1978.)

to test the hypothesis that the prevalence rate of infection among ovipositing females is higher than that among females of the following generation (because only a proportion of the eggs laid by an infected female carry virus), and that, consequently, amplification within vertebrate hosts is required for perpetuation of the virus (Figure 41.4). Their more advanced models concerned particular phases of the transmission cycle. In the absence of much essential quantitative field data, these models of KEYV transmission have not been tested, so the concepts of transmission that they explore remain hypothetical.

41.5 VIRULENCE

41.5.1 Characteristics

In this section, the term 'parasite' is used for any form of infective agent. Where in nature a parasite population interacts with a population of its natural host, the extent of harm caused to infected individuals is an important factor in the dynamics of both populations. Two descriptive terms pertinent to such situations are: **pathogenicity** – the ability to cause pathological changes or disease; and **virulence** – the degree of pathogenicity of a parasite as indicated by the cruelty of the disease produced. Parasitologists are likely to treat pathogenicity and virulence as aspects of host/parasite interaction that result in costs to the fitness of both parasite and host. Virulence is the more useful of these terms – all parasites, as distinct from other symbionts, are necessarily pathogenic. In epidemiological studies, virulence may be one variable, but often any quantitative measurements of virulence are crude, e.g. the rates of morbidity or death in host populations.

What is usually perceived as a pathological state or a disease of the host is the consequence of modifications of the host induced in it by the parasite to its own advantage, to enable it to survive, develop and multiply within the host. Not all modifications of the host are pathological; some may reduce the reproductive capability of the host. These actions of the parasite are genetically regulated. The perceived virulence of a parasite population is an aggregate of the mechanisms that make up its disease-producing capability. Infection of a host triggers a response in the form of activation of innate immune mechanisms, which also are genetically regulated (Chapter 42). The refractoriness of a host population is an aggregate of the defensive mechanisms employed against the parasites infecting it. Where in nature populations of a host and a parasite occur together, the interactions between them generate selective forces that affect the virulence of the parasites and the refractoriness of the hosts, with the possibility of modification of either; in other words, there is a trade-off between those two variables (Section 42.1.2). This is usually perceived as a trade-off between the aggressiveness of the parasites and the diseased state of the hosts.

Such interactions have been extensively modelled, but we examine just the equation for

the basic reproductive rate, which was devised for measurement of the transmission of malaria parasites (Macdonald, 1955) and developed for analysis of the population dynamics of micro-parasites (Anderson and May, 1981). Estimates of the basic reproductive rate of myxoma virus were used by Anderson and May (1982) in their study of interactions between myxoma virus and populations of the European rabbit in Australia, where mosquitoes served as vectors through mechanical transmission (Section 43.4.3.b; Eqn 43.2). Here, that equation is elaborated and the notation modified as suggested by Frank (1996).

$$R_0 = \frac{\beta(v)N}{\delta + v + c(v)} \qquad (41.10)$$

where R_0 is the basic reproductive rate of the parasite, δ is the host's disease-free death rate, v is the disease-induced mortality rate (virulence), c is the rate at which hosts recover by clearing the infection, β is the rate of transmission of the disease between infected and susceptible hosts, and N is the total population size of the host.

The terms in the equation have simple intuitive meanings. Transmission, $\beta(v)N$, is the number of new infections per unit time produced by one infected individual introduced into a population of N uninfected hosts. The denominator terms, $1/[\delta + v + c(v)]$, describe how long an infection is expected to persist within a host: the host may die as a result of parasite virulence, v; or the infection may be cleared by the host, $c(v)$; or the host may die of other causes, δ. The product of transmission and residence time in the host determines the total number of new infections emanating from an infected host. Equation 41.10 indicates that a higher rate of transmission increases parasite fitness, whereas higher virulence decreases parasite fitness because it damages the parasites' food supply (the host). Parasite fitness is governed by the balance between the benefits of high transmission and the costs of increased virulence (Frank, 1996).

As noted in the following section, selection does not produce a balance between increasing success of the parasite and increasing costs to the host, resulting in intermediate levels of virulence. Nor do host–parasite relationships evolve to zero virulence with no disease, because the functional causes of virulence are beneficial to the parasite, increasing its transmission success during its lifetime. However, virulence can carry a cost in the form of premature host death, which shortens the lifetime of the infection. Consequently there is a trade-off between how fast and for how long the parasite transmits. This is thought to result in maximum 'lifetime transmission' occurring in parasites that cause an intermediate degree of virulence. Selective forces may drive virulence upwards, but only to the point where reductions in transmission caused by host death begin to outweigh the benefits of transmission (Frank, 1996).

A very large epidemiological survey, the Garki Project, was carried out in a malaria-endemic area in Nigeria where *P. falciparum* predominated (Molineaux and Gramiccia, 1980). Later analysis of the field data revealed strong positive correlations between asexual multiplication, transmission rate and infection length, and host morbidity and mortality. The expected total lifetime transmission of the parasite was maximal in young children, in whom the fitness cost to the parasite of host mortality balanced the fitness benefits of higher transmission rates and slower clearance rates, a clear example of trade-off (Mackinnon and Read, 2004).

In cases of direct transmission, i.e. of a pathogen from one host individual to another of the same species, it is necessary that the infective hosts maintain their motility; and for that reason, it is thought, selection acts against any exploitation of a host that causes severe pathogenicity. In cases of indirect transmission in which the two hosts are a vertebrate and a haematophagous arthropod, the latter being the vector, both hosts are liable to suffer pathogenic effects, but selective forces act differently on the two. It is not essential for transmission that infective vertebrate hosts retain their motility, therefore intensive exploitation of the vertebrate host should carry lower costs to the pathogen than in the case of directly transmitted

pathogens. In contrast, the continued motility of infected vectors and protection of their lifespans are essential for transmission. Within populations of humans at risk of infection with some 60 infective agents (viruses, bacteria or protozoans), mortality rates were markedly and significantly higher with the vector-borne infective agents than with those that were directly transmitted (Ewald, 1983; Ewald and De Leo, 2002).

41.5.2 Genetic variability and evolution

Microparasites have short generation times, and usually show considerable genetic variability; therefore, natural selection can cause rapid changes in the genetic make-up and phenotypic characteristics of their populations. Vertebrate hosts have much longer generation times, and so can respond only relatively slowly to parasite-imposed selection pressure by developing resistance. Both parasites and hosts exploit their genetic heterogeneity to adapt, and eventually evolve, in response to one another and to their environment.

For many years it was thought that parasites that harm their hosts have reduced chances of survival and that, in consequence, parasitic species that cause serious disease are disadvantaged over time. Individual traits may have positive, neutral or negative effects on the fitness of a parasite or a host. It was predicted that selection acting on such traits would produce a balance between increasing success of the parasite and increasing costs to the host – leading to its death, and resulting in intermediate levels of virulence. That was shown not to be the case, notably with myxoma virus infecting rabbits (Section 43.4.3), and the view long held by parasitologists that all parasites evolve to become mild for their hosts has been rejected (Bull, 1994; Sabelis and Metz, 2002). Observational evidence and modelling have shown that host/parasite relationships do not develop to benignity on short time scales; nor do they evolve towards it on evolutionary time scales.

Equally, host–parasite relationships do not evolve to zero virulence with no disease, because the functional causes of virulence are beneficial to the parasite, increasing its transmission success during its lifetime. For example, by extracting more resources from the host, the parasite is able to produce more of its transmissible forms per unit time. Also, where a mechanism that reduces clearance of a parasite by its host becomes enhanced, the duration of infection is longer so increasing lifetime transmission. The idea that a well-adapted parasite should necessarily cause only slight or moderate virulence is now regarded as erroneous. Theory and empirical evidence support the idea that natural selection can drive a host–parasite relationship to any position along the range between commensalism and lethality (Ewald, 1994, 1995; Ewald and De Leo, 2002).

Empirical observation shows virulence to continue in many host–parasite relationships of very long standing, but the phenomenon that hosts are not severely affected by infection (Section 41.1.2) suggests that virulence can decline to a maintained, relatively harm-free level. Two examples that involve mosquitoes as vectors indicate that, on a time scale longer than historical time, some vertebrate–pathogen associations have evolved to a relatively harm-free state. (i) The effects of infection with yellow fever virus on African monkeys are mild, and few infected monkeys die. In contrast, among New World monkeys, first exposed to yellow fever virus in the 16th century, species of *Alouatta*, *Saguinus* and *Aotus* still suffer high fatality rates (Sections 45.3.2.b; 45.3.6.c). (ii) After the arrival of *Culex quinquefasciatus* in the Hawaiian Islands in 1826, epizootics, first of 'bird pox' and later of avian malaria due to *Plasmodium relictum capistranoae*, led to the decline of many native bird species and the local extinction of others. Introduced species of birds were relatively tolerant of infection (Section 51.9.2).

Attempts to elucidate the evolutionary history of host–parasite relationships are fraught, because the evidence must be based on a variety of approaches. Two examples concerning *P. falciparum*, both cited from Rich and Ayala (2000), illustrate that point. Divergence between *Plasmodium reichenowi*, the chimpanzee parasite, and *P. falciparum*, the human parasite, is estimated to have occurred 8–12 million

years ago, which is roughly consistent with the time of divergence between the two host species, chimpanzees and humans. This suggests that *P. falciparum* is an ancient human parasite, associated with our ancestors since the divergence of humans from the great apes. Another approach to elucidating the evolutionary relationships of *P. falciparum* involved silent (i.e. synonymous) nucleotide polymorphisms, which can be used to estimate the age of genes because they reflect the mutation rate and the time elapsed since their divergence from a common ancestor. That approach revealed a scarcity of synonymous polymorphisms in ten *P. falciparum* genes. Of five possible explanations, the most satisfactory pointed to a recent population bottleneck, indicating that extant world populations had been recently derived from a single ancestral strain (Rich and Ayala, 2000).

42

Immune responses of mosquitoes

This chapter contains descriptions of the types of immune response to infection found in mosquitoes. The descriptions are cross referenced to the sections of other chapters that include more detailed accounts of the responses to particular pathogens or parasites.

42.1 INTRODUCTORY MATTERS

42.1.1 Types of immunity

Two types of immune mechanism are recognized – innate and specific. **Innate immune mechanisms**, which function in insects as in other animals, involve a variety of defences, including release of antimicrobial peptides and phagocytosis by blood cells. They are activated within seconds of encountering an elicitor. The responses to invasive organisms recognized as 'non-self' are relatively non-specific and no cellular memory is required. 'Pattern recognition receptors', which trigger responses, do not recognize unique antigens but are specific for a few, highly conserved structures that are present in large groups of potential pathogens, e.g. the lipopolysaccharides of Gram-negative bacteria and lipotechoic acids of Gram-positive bacteria. For insects, which have a relatively short lifespan, this is the more effective form of defence. **Specific (or adaptive) immune mechanisms**, found in vertebrates, involve the highly specific recognition of foreign antigens by means of immunoglobulins, major histocompatibility complex molecules and T-cell receptors. A cellular memory provides the ability to raise an enhanced response, as with antibodies, when a pathogen is re-encountered. It is possible that, within their limitations, insect immune systems have greater capabilities than is generally accepted. Putative cases of cellular memory in insects, in which the hosts raised an enhanced immune response following an initial encounter with an elicitor, are discussed by Pham and Schneider (2008).

The innate immune mechanisms of insects share some similarities with those of mammals, but they will be mentioned only very briefly. Our knowledge of the insect immune system largely derives from genetic studies on *Drosophila*

melanogaster and data obtained from a few lepidopteran species such as *Manduca sexta*, the size of which make biochemical studies feasible. Comparison of the innate immune mechanisms found in *Anopheles gambiae* with those known from *D. melanogaster* have proved informative.

The immune responses of mosquitoes can be very effective in countering invasion by prokaryotic and eukaryotic parasites. They involve humoral and cellular components and combinations of both. The humoral component includes inducible antimicrobial peptides and a phenol oxidase cascade system that both yields reactive oxygen and nitrogen intermediates and is involved in encapsulation. The cellular component involves haemocytes that are involved in phagocytosis or in encapsulation. Most investigations into the immune responses of mosquitoes have concerned parasites that infect both humans and mosquitoes – namely malaria parasites and filarial nematodes of which mosquitoes are hosts and vectors. Antimicrobial peptides have been studied mostly for their antibacterial activity.

42.1.2 Comparison of immune responses observed in laboratories and in nature

Where, in nature, populations of a host and a parasite occur together, the interactions between them generate selective forces that affect the virulence of the parasite to the host and the refractoriness of the host to the parasite. There is a trade-off between those two – the virulence and the refractoriness. A host mosquito invests resources into its immune mechanisms, while the parasite invests resources into suppressing or evading those immune responses. Both investments have a cost, the cost to the host being measurable in the reduction in reproductive success.

Koella and Boëte (2003) developed a mathematical model of the coevolution of immunity and immune evasion based on perceived interactions between populations of malaria parasites and their mosquito hosts. The model was considered appropriate also for other parasites that are indirectly transmitted by intermediate hosts. Three outcomes of the interactions between parasite and host populations were possible. First, a stable equilibrium with intermediate levels of investment by host and parasite, both partners investing less as the intensity of transmission increases. Second, where the cost of investing into resistance is sufficiently high and transmission is sufficiently intense, the host invests no resources in its immune response and, accordingly, the parasite invests nothing in a counter-response. Third, where the cost of immunity is high and transmission is intermediate, no stable equilibrium is achieved but the levels of investment by host and parasite cycle around intermediate levels.

Most investigations into mosquito immune systems are undertaken in laboratories distant from natural mosquito populations. Often model systems are used, i.e. host/parasite associations that do not occur in nature but that can be produced in the laboratory. Even if host/parasite associations that occur in nature are used in the laboratory, there has been no continuous association between the colonized hosts and the parasites that permitted trade-offs between them. Comparisons between findings from the laboratory and field are enlightening. When laboratory-reared *Anopheles gambiae* were infected with *Plasmodium falciparum*, typically many oocysts formed in each female and most became encapsulated (Collins *et al.*, 1986). In contrast, in regions of Africa where malaria was holoendemic, in wild-caught females of *An. gambiae* infected with *P. falciparum* very few parasites survived the hosts' defences to develop to the oocyst stage, and of those oocysts none or only very few were encapsulated (Hogg and Hurd, 1997; Taylor, 1999; Schwartz and Koella, 2002).

Experimental infection of *An. gambiae* with the rodent parasite *Plasmodium berghei* (not a natural combination) led to the formation of many oocysts, most of which matured and produced sporozoites. The roles of three recognition proteins (CTL4, CLTMA2 and LRRM1) were examined by gene silencing. Knockout of the genes for CTL4 or CTLMA2 resulted in the encapsulation of high percentages of the parasites, indicating that those proteins normally protect malaria parasites against encapsulation. In contrast, knockout of the gene

for LRRM1 led to a 3.6-fold increase in oocyst numbers with no encapsulations, suggesting that LRRM1 is normally involved in the killing of a substantial proportion of invading ookinetes, possibly by lysis, before oocyst formation (Osta *et al.*, 2004). Hemingway and Craig (2004) asserted that those findings were 'the clearest example yet of coevolution between the parasite and its insect vector'. The investigation was continued in Cameroon, where it involved colonized females of the local Yaoundé strain infected by feeding on *P. falciparum* gametocyte carriers in Mfou, 30 km from Yaoundé city. Knockout of the genes for CTL4, CTLMA2 or LRRM1 had no significant effect on development of the parasites. Whether the differences in outcomes between the laboratory and field investigations were due to the difference in species of malaria parasite or a result of coadaptation of the genotypes of the parasite/vector populations was uncertain (Cohuet *et al.*, 2006).

Findings from laboratory investigations into the immune responses of mosquitoes must be interpreted with caution. They can identify the different immune processes that are available to the mosquito, and they can elucidate the mechanisms by which those processes function, but they do not reveal the relative importance of the different immune processes in wild mosquitoes or permit the possibility of trade-off between mosquito and parasite populations.

42.1.3 Evolutionary genetics of innate immune systems

To explore the evolutionary dynamics of innate immunity in *D. melanogaster*, *An. gambiae* and *Stegomyia aegypti*, of which the complete genomes had been analysed, Waterhouse *et al.* (2007) undertook a meta-analysis of data concerning 31 gene families and functional groups implicated in innate immunity or defensive functions. Bioinformatic analysis identified 4951 orthologous trios of genes in the three species, and 886 'mosquito-specific' orthologous pairs of genes that were absent from *Drosophila*. Combined bioinformatic analysis and 'manual curation' of the immune repertoire identified 91 trios and 57 pairs, plus a combined total of 589 paralogous genes in the three species. Probably, orthologues serve corresponding functions whereas paralogues may have acquired different functions. Plotting phylogenetic distances between orthologous immune genes in the *Drosophila–St. aegypti* pair and in the *Drosophila–An. gambiae* pair revealed, on average, significantly greater divergence between the orthologous immune genes of that trio of species than between the totality of trios of orthologous genes in their collective genomes.

Inter-species divergence was illustrated with two types of recognition receptor that belong to distinct structural classes – thioester-containing proteins (TEPs) and leucine-rich repeat proteins (LRRs). Members of both classes are associated with the killing and disposal of parasites by lysis or encapsulation (Section 42.4). AgTEP1, for example, binds to the surface of both *Plasmodium* and bacteria. Bioinformatic analysis showed that TEPs form one orthologous trio and two groups: one group includes *Drosophila* and mosquito peptides, while the other comprises only mosquito species-specific clades. The three LRR proteins that recognize malaria parasites are produced by *An. gambiae* but not by *St. aegypti* (which is not a natural host of malaria parasites) or *Drosophila*. From this and other evidence, Waterhouse *et al.* (2007) concluded that the acquisition of new functions for recognition proteins, such as recognition of malaria parasites, is through the modification of genes bearing powerful and ancient recognition domains.

42.2 IMMUNE RESPONSES TO VIRUSES

Infection by a virus involves penetration of cells, replication and dissemination to other tissues. The first step in cell penetration is the binding of 'attachment protein' molecules on the surface of the virus to specific 'receptor' molecules on the cell's surface. Once bound, viruses may be internalized by a variety of mechanisms (Section 43.1.1). The concept of non-structural barriers to

invasion or dissemination of viruses has some support in the descriptively named 'midgut infection barrier' and 'midgut escape barrier' (Section 44.8.3.a), but their mechanisms are not known.

When the natural mammalian hosts of arboviruses become infected they either die or recover and remain immune for life. In contrast, the mosquito hosts of the same viruses remain infected for the rest of their lives. Examples in which this has been established include *St. aegypti* infected with dengue virus, *Culex univittatus* infected with West Nile virus (WNV) and *Haemagogus janthinomys* infected with yellow fever virus (Section 44.8.1.g). If such lifelong persistence is characteristic of all infections of mosquitoes with an arbovirus, it follows that the innate immune system of mosquitoes is incapable of eliminating arboviruses. However, there is evidence that mosquitoes can reduce the amount of virus in their bodies.

Oral infection of *St. aegypti* with Sindbis virus induced the Toll pathway-related Rel1 transcription factor (Section 42.4.2) in midgut tissue (Sanders *et al.*, 2005). Infection of *St. aegypti* with DENV-2 induced a set of genes corresponding to the Toll pathway. Activation of Toll and Imf pathways in *St. aegypti* through RNAi-mediated silencing of Cactus and Caspar caused a reduction in the extent of infection with dengue virus that appeared to be controlled primarily by the Toll pathway. Repression of the Toll pathway through MyD-gene silencing resulted in higher dengue virus infection levels. Activation of the Toll pathway was supported by the up-regulation of Spaetzle (Spz), Toll and Rel1A, and the down-regulation of the negative regulator Cactus. The results suggested that the infection of mosquitoes with DENV-2 induces the Toll pathway, which then exerts an anti-dengue effect (Xi *et al.*, 2008).

RNA interference (RNAi), once termed 'post-transcriptional gene silencing', is a pathway that enables cells to control which genes are active and how active they are. Two types of small RNA molecules are central to RNAi, namely microRNA (miRNA) and small interfering RNA (siRNA). They can bind specifically to some other RNAs and either increase or decrease their activity. The RNAi pathway is initiated by short, double-stranded RNA (dsRNA) molecules in a cell's cytoplasm, which interact with argonaute, a component of the 'RNA-induced silencing complex' (RISC).

Most mosquito-borne arboviruses are ssRNA (single-stranded RNA) viruses (Table 43.1). However, replication of the ssRNA O'nyong-nyong virus (*Togaviridae*, *Alphavirus*) in infected BHK cells led to replicative intermediates, i.e. dsRNA forms. Following inoculation of *An. gambiae* with O'nyong-nyong virus (ONNV) that had been engineered to carry a GFP (green fluorescent protein) marker, the virus spread to other tissues over a 9-day incubation period. When the ONNV-eGFP was co-inoculated with dsONNV, the ONNV-eGFP titres were significantly lower after 3 and 6 days. But when ONNV-eGFP was co-inoculated with dsRNA from *AgAgo2*, a gene that silences RNAi, the virus titres were 16-fold higher than the controls at 3 and 6 days. Keene *et al.* (2004) concluded that RNAi is an antagonist of ONNV replication in *An. gambiae*.

Dengue virus (DENV) (*Flaviviridae*, *Flavivirus*) is another ssRNA virus. Although oral infection of *St. aegypti* with DENV2 generated dsRNA and production of DENV2-specific siRNAs, virus replication and release of infectious virus persisted, suggesting viral circumvention of RNAi. However, silencing certain genes in the RNAi pathway (*dcr2*, *r2d2*, *ago2*) increased virus replication in the host and decreased the extrinsic incubation period required for virus transmission. Sánchez-Vargas *et al.* (2009) concluded that RNAi is a major determinant of DENV transmission by *St. aegypti*.

RNAi functions not only in regulating gene expression but also as an innate defence mechanism, protecting cells against viruses and transposons; experimental evidence has shown that the RNAi pathway is present and active in anopheline and culicine mosquitoes. When initiated, it leads to the destruction of any mRNA that has sequence identity with the dsRNA trigger. After inoculation of *An. gambiae* with o'nyong-nyong virus (ONNV) that had been engineered to

carry a green fluorescent protein (GFP) marker, the virus was seen to spread to other tissues. When the ONNV-eGFP was co-inoculated with dsRNA from *AgAgo2*, a gene that silences RNAi, the virus titres were 16-fold higher than the controls at 3 and 6 days. Keene *et al.* (2004) concluded that RNAi is an antagonist of ONNV replication in *An. gambiae*. Silencing certain genes in the RNAi pathway (*dcr2*, *r2d2*, *ago2*) increased DENV-2 replication in *St. aegypti*, leading Sánchez-Vargas *et al.* (2009) to conclude that RNAi is a major determinant of DENV transmission by *St. aegypti*.

42.3 SERINE PROTEASES

References are made in this and some other chapters to serine proteases that are involved in immune responses or developmental processes. Some proteins, including enzymes, require activation before they can function – activation involving limited proteolysis of a precursor molecule by a serine protease. In many cases, the activity of serine proteases is regulated by serpins, a class of protease inhibitor.

42.3.1 Characteristics

Serine proteases are members of a large family of extracellular endopeptidases in which the active centre contains a catalytically active serine residue. When these enzymes cleave peptide bonds, an ester is briefly formed between the hydroxyl group of the active serine and the carboxyl group of the cleaved peptide bond.

Some serine protease molecules contain a domain of approximately 30–60 amino acids that includes three disulphide bonds which give it a 'paperclip'-like configuration. They are termed 'CLIP-domain serine proteases' (clip-SPs) and function in developmental and immune processes. The CLIP domain is thought to regulate the activity of the catalytic-protease domain. From *An. gambiae* two classes of CLIP have been defined: class CLIPA, which consists of ten catalytically inactive putative serine protease homologues (CLIPA1–10); and class CLIPB, which consists of 17 catalytically active enzymes (CLIPB1–17). In contrast, the serine proteases of *St. aegypti* include many fewer CLIPAs and many more CLIPBs. In *An. gambiae*, activation of phenol oxidases by limited proteolysis of their pro-phenol oxidase zymogens is induced by a protease cascade, mostly of CLIPBs, which is regulated, positively and negatively, by a network of inactive protease homologues – CLIPAs, CTLs (C-type lectins) and SRPNs (serine proteinase inhibitors). Reverse genetic analyses identified a set of regulators of the melanin synthesis associated with encapsulation of *P. berghei* or of Sephadex beads (viz. one SPRN, two CTLs, three CLIPAs and eight CLIPBs) (Waterhouse *et al.*, 2007, review).

Five serine proteases were identified as haemolymph proteins of *An. gambiae*. Four were CLIP proteases, of which three (Sp14A, Sp14D1, Sp14D2) showed changes in transcript abundance following immune challenge, and one (Sp18D) showed no such response. The fifth serine protease, Sp22D, was expressed constitutively in adult haemocytes, fat body cells and midgut epithelial cells. It had a complex multi-domain structure including a trypsin-like serine protease domain, two putative chitin-binding domains, a mucin-like domain, and two cysteine-rich domains (Gorman *et al.*, 2000a,b; Gorman and Paskewitz, 2001).

The actions of proteases are restricted to specific functions by protease inhibitors, notably serpins. Serpins constitute a protein superfamily of proteins, many of which are serine proteinase inhibitors (SRPNs). Serpins bind tightly to activated proteases, blocking their activity.

42.3.2 Functions

Serine proteases are known to function in the immune responses of mosquitoes by activation of recognition proteins, activation of antimicrobial peptide synthesis and activation of certain phenol oxidases involved in the biosynthesis of melanin. In their various life-cycle stages malaria parasites also make use of serine proteases, for example in effecting secondary severance of surface proteins (Volume 4, Chapter 51).

Shortly after septic injury of *An. gambiae*, the recognition protein TEP1 is proteolytically cleaved

into a small and a large fragment; both fragments remain associated as a single, two-chain molecule. The carboxy-terminal fragment contains a conserved thioester site that covalently attaches the molecule to the surface of $Gram^+$ and $Gram^-$ bacteria, labelling them for clearing by phagocytosis (Levashina *et al.*, 2001). In *An. gambiae*, most of the serine proteases that are transcriptionally upregulated after infection by *Plasmodium berghei* belong to the CLIP family, several members of which are believed to cause signal amplification (Barillas-Mury, 2007).

In adult *An. gambiae*, *Sp14D1* and *Sp14D2* showed slight upregulation after injection of saline or bacteria, or infection with malaria parasites; *Sp14A* showed upregulation only after infection with malaria parasites. Abundance of the *Sp22D* transcript was unaffected by injection of saline or infection with *Plasmodium*, but it increased 1.5-fold after injection of bacteria (Gorman and Paskewitz, 2001).

42.4 RECOGNITION PROTEINS

Conceptually, the innate immune reaction of insects can be divided into four steps: (i) recognition of non-self, microbial molecules of a variety of chemical classes and binding of host 'recognition proteins' to ligands on those molecules; (ii) modulation, leading to amplification and spread of the initial recognition signal; (iii) activation of a diverse suite of effector processes; and (iv) replenishment of immune-related molecules via activation of signal transduction pathways. Signal amplification results from the proteolytic action of serine proteases, regulated by serpins.

42.4.1 Characteristics of recognition proteins

Immune reactions start with contact between host recognition proteins, which may be soluble or cell-bound, and invading microorganisms that are characterized by any of a variety of conserved 'pathogen-associated molecular patterns' (PAMPs). 'Pattern-recognition receptors' borne on recognition proteins recognize and bind to PAMPs that are common on microorganisms but rare in or absent from the host species. Such non-self molecules that are present in the cell walls and membranes include peptidoglycans and lipopolysaccharides of bacteria and β-1,3 glucans of fungi. The non-self molecules that are recognized on the surface of malaria parasites and filarial nematodes remain little known. Upon binding, pattern recognition receptor molecules can either directly mediate microbial killing through encapsulation and phagocytosis or can trigger a variety of defence reactions through the activation of serine protease cascades and intracellular immune signalling pathways that control the transcription of effector genes, such as those for antimicrobial peptides (Warr *et al.*, 2008).

Genes that encode recognition proteins were identified in the *An. gambiae* genome, revealing six families of recognition proteins, namely: (i) peptidoglycan recognition proteins (PGRPs); (ii) thioester-containing proteins (TEPs); (iii) Gram-negative bacteria-binding proteins (GNBPs); (iv) C-type lectins (CTLs); (v) leucine-rich repeat proteins (LRRs); (vi) scavenger receptor proteins (SCRs); and (vii) galectins (GALEs) (Christophides *et al.*, 2002; Osta *et al.*, 2004; Michel and Kafatos, 2005). An expression screen for immune response genes in the genome of adult female *An. gambiae* revealed that its immune system discriminates between molecular signals that are specific to infection with bacteria or malaria parasites (Oduol *et al.*, 2000). Certain recognition proteins are opsonins, i.e. proteins that, when bound to a microorganism, increase its susceptibility to phagocytosis by linking the surface of the microorganism to a specific receptor on the surface of a phagocytic cell.

In mosquitoes, the thioester-containing complement-like protein TEP1 and two leucine-rich repeat proteins, LRIM1 and APL1, are major factors in the regulation of parasite loads. TEPs are characterized by homologous sequences, including a unique β-cysteinyl-γ-glutamyl thioester bond that mediates covalent attachment of the molecules to activating self- and non-self surfaces. Proteolytic activation of a TEP molecule generates a two-chain molecule consisting of a small and a large fragment, the larger binding covalently to the target

cell surface through hydrolysis of the thioester. Binding of activated TEP1 to pathogens targets them for phagocytosis or destruction by lysis. LRIM1 and APL1 are required for the binding of TEP1 to parasites; they first stabilize each other and subsequently stabilize circulating TEP1. These three antiparasitic factors jointly function as a complement-like system in parasite killing, the two LLR proteins serving as complement control factors (Levashina *et al.*, 2001; Blandin and Levashina, 2004a,b; Fraiture *et al.*, 2009).

The opsonizing binding of TEP1 to the surface of $Gram^+$ and $Gram^-$ bacteria was thioester-dependent. Chemical inactivation of the thioester inhibited the phagocytotic activity of a mosquito haemocyte-like cell line *in vitro*. TEP1 bound to the surface of *P. berghei* ookinetes in the basal labyrinth of the midgut epithelium, promoting their killing by lysis. At 11 days post-infection, well-developed oocysts were covered with TEP1, but this binding did not interfere with the parasite development. In susceptible mosquitoes, knockout of *TEP1* resulted in a fivefold increase in the number of oocysts developing on the midgut (Levashina *et al.*, 2001; Blandin *et al.*, 2004; Blandin and Levashina, 2004b).

42.4.2 Actions of recognition proteins

(a) Actions during the pre-invasion phase

Infections of *An. gambiae* by *P. berghei* can conceptually be separated into two phases: (i) a pre-invasion phase that extends from oral intake of the parasite into the midgut lumen until the moment of penetration of ookinetes into the midgut epithelium; and (ii) a post-invasion phase which extends from ookinete penetration into the midgut epithelium onwards. The mosquito's immune responses comprise constitutive elements, which are expressed before infection, and reactive elements, which are expressed after the contact of recognition proteins with the parasite.

The constitutive immune response is regulated predominantly by the transcription factors Rel1 and Rel2, members of the Rel protein family (Table 42.1). As a result of their activity, and before infection, haemocytes constitutively secrete the recognition proteins TEP1, APL1 and LRIM1 into the haemolymph, and these molecules accumulate in the basal labyrinth of cells of the midgut epithelium. Concurrent silencing of the transcription factor genes *REL1* and *REL2* decreased the basal expression of the genes that encode

Table 42.1 Regulatory components of the immune systems of *Anopheles gambiae* and *Stegomyia aegypti*, with details of the signal transduction pathways and transcription factors. Comparable data for *Drosophila melanogaster* are also provided, with the names of orthologous transcription factors. (From the data of Barillas-Mury *et al.*, 1996; Meister *et al.*, 2005; Shin *et al.*, 2005; Frolet *et al.*, 2006.)

Signal transduction pathways	*Orthologous transcription factors*		
	An. gambiae	St. aegypti	D. melanogaster
	NF-κB-like class	*NF-κB-like class*	*NF-κB-like class*
Toll	Rel1*	AaRel1	Dorsal (D1)
Imd	Rel2	AaRel2	Relish
Toll	–	–	Dif †
	IκB-like class	IκB-like class	IκB-like class
Toll	Cactus	Cactus	Cactus ‡

*, Rel1 of *An. gambiae* was originally named Gambif1.
†, Dorsal-related immunity factor (Dif).
‡, Phosphorylation of Cactus removes its inhibition of Dorsal and Dif.

TEP1 and LRIM1 and abolished resistance of *An. gambiae* to *P. berghei*. These findings demonstrate the crucial role of the pre-invasion defence in the elimination of parasites, which is, at least in part, based on molecules circulating in the haemolymph (Meister *et al.*, 2005; Frolet *et al.*, 2006).

Transcription of immune genes occurs constitutively in newly emerged mosquitoes. During the pre-invasion phase, silencing of either *REL1* or *REL2* alone did not substantially affect parasite survival, but a double knockout of *REL1* and *REL2* decreased the constitutive expression of *TEP1* and *LRRM1* by 70%, and resulted in a two-fold increase in the number of parasites developing in the host midgut. These results indicate that the pre-invasion phase is characterized by expression of *TEP1* and *LRRM1*, so-called basal immunity, regulated by Rel1 and Rel2 (Frolet *et al.*, 2006).

Silencing the gene encoding the negative regulator Cactus boosted pre-invasion gene expression; thus, *TEP1* expression was markedly increased within 12 h after injection of *dsCactus*, and a strong *TEP1* signal was observed in haemocytes. This increased expression was strictly dependent on Rel1 upregulation of *TEP1* transcription, but not that of Rel2. The constitutive expression of *LRIM1*, *APL1* and *CTL4* was also upregulated by depletion of Cactus in a Rel1-dependent manner (Frolet *et al.*, 2006; Riehle *et al.*, 2008).

In *An. gambiae*, the pre-invasion period is characterized by basal expression of the proteins TEP1, APL1 and LRRM1. Haemocytes constitutively secrete these proteins into the haemolymph, and they accumulate within the basal labyrinth of the midgut epithelium. Invasive malaria parasites are exposed to constitutively produced recognition proteins when they first come into contact with the haemolymph. TEP1 produced in mosquito haemocytes is probably always circulating in the haemolymph, and can bind to the surface of invading parasites when they reach the basal side of the midgut epithelium, inducing their killing. Use of immunofluorescence revealed TEP1 in haemocytes during the period up to 18 h post-infection, the time when ookinetes first invaded midgut cells. At 24 h post-infection the haemocytes appeared devoid of TEP1, suggesting massive secretion (Levashina *et al.*, 2001; Blandin *et al.*, 2004, 2008).

(b) Actions during invasion and during the post-invasion phase

Some 20–30 h after *An. gambiae* had ingested *P. berghei*-infected blood, about the period when ookinetes invade the midgut epithelium, a number of genes encoding putative defence molecules are induced. Increased levels have been observed of mRNAs encoding the recognition protein GNBP and the antimicrobial peptide defensin. The mRNA titres increased in the invaded gut tissue and in the rest of the carcass, most probably in the fat body and haemocytes. Six members of the *GNBP* gene family function in the innate immune system of *An. gambiae*. Of those, *GNBP1* is mainly expressed in the salivary glands, while *GNBP4* is a major factor in defence against a broad range of pathogens, including the Gram$^-$ *E. coli* and *P. berghei* (Warr *et al.*, 2008).

During the invasion of midgut cells, expression of *TEP1* is induced by an unknown factor, and at the start of the post-invasion period there is a marked increase in transcription of *TEP1*, *LRR1* and *CTL4*, and in the synthesis of their expression products, recognition proteins in haemocytes. Transcription of *TEP1* was upregulated by three-fold at 24 h post-infection, but this was transient, and by 48 h post-infection the titre of TEP1 had fallen back to the initial, pre-infection amount (Osta *et al.*, 2004; Frolet *et al.*, 2006; Blandin *et al.*, 2004, 2008).

The protein APL1 (*Anopheles Plasmodium* responsive leucine-rich repeat 1) functions in the immune response to malaria parasites. It is a product of *APL1*, a family of at least three independently transcribed genes, *APL1A*, *APL1B* and *APL1C*, of which *APL1C* was found to function in the innate immune system. Gene silencing in *An. gambiae* showed that APL1C activity is necessary to provide a high level of protection against *P. berghei* infection. APL1C functions within the Rel1-Cactus immune signalling pathway, which regulates *APL1C* transcription and abundance of the protein. Silencing *APL1C* abolished Rel1-mediated protection against

P. berghei. In the laboratory, *APL1C* functioned as a transducer of Rel1-dependent immune signals, efficiently protecting *An. gambiae* from infection with *P. berghei*. Re-sequencing of parts of the *APL1* genes identified a high level of polymorphism that was characteristic of different strains of *An. gambiae*, and was correlated with the variable susceptibility of those strains to infection (Riehle *et al.*, 2008). Another leucine-rich repeat (LRR) domain protein is sometimes named APL2, but more frequently LRRD7 (Dong *et al.*, 2006; Riehle *et al.*, 2006).

42.5 REGULATION OF THE IMMUNE RESPONSE IN *ANOPHELES GAMBIAE*

Most investigations into the regulatory mechanisms that govern the immune responses of mosquitoes have concerned the oral infection of *An. gambiae* with *P. berghei* or, to a lesser extent, responses to the experimental inoculation of bacteria, usually *Micrococcus luteus* and *Escherichia coli*. Malaria parasites that are ingested by vector mosquitoes are exposed to two sequences of immune response, which may be termed 'constitutive' and 'reactive'. (i) Newly emerged, uninfected females constitutively transcribe certain immune genes whose expression products have a crucial role in the elimination of parasites. This is a phase of constitutive or basal expression. (ii) At different stages after their ingestion the parasites trigger the upregulation of immune-response genes, initiating a period of reactive expression. This may start when parasites are in the gut lumen (Volume 4, Section 51.9), and it occurs strongly when ookinetes invade cells of the midgut epithelium and reach the basal lamina, where they become exposed to haemolymph. Bacteria that penetrate into the haemocoel through wounds in the integument are immediately exposed to constitutively expressed immune processes in the haemolymph and trigger reactive responses which may quickly take effect. Both the constitutive and the reactive immune responses to infection involve transcription factors, which regulate the expression of immune genes, including those expressing antimicrobial peptides.

The signal transduction pathways of *Drosophila melanogaster* were investigated before those in mosquitoes, and the names of certain constituents in *Drosophila* were later adopted for the homologous structures in mosquitoes. The practice of describing *Anopheles* genes as, for example, 'unambiguous orthologues of their *Drosophila* counterparts' might give the impression of *Drosophila* as being ancestral. In fact, many genes with immune functions that were present in the most recent common ancestor of those two genera were conserved in the Culicidae before the Drosophilidae arose.

42.5.1 Signal transduction pathways

Signal transduction pathways link the first stage of an immune response, recognition of non-self molecules and amplification of the recognition signal, to the transcriptional activation of effector substances, including antimicrobial peptides. The better-known signal transduction pathways of ***Drosophila melanogaster***, named Toll and Imd (immunodeficiency), are described here first. These pathways function in embryonic development and in immune responses to fungi and bacteria. Toll is a transmembrane protein. Its extracellular domain consists of leucine-rich domains which interact with the ligand named Spaetzle. The intracellular domain interacts with the proteins MyD88, Tube and Pelle, probably forming an inactive protein kinase complex. The key elements of the antifungal defence of *Drosophila* are Spaetzle, Toll, the transcription factor Dif and its inhibitor Cactus (Table 42.1).

Within fat body cells, the Toll pathway of *Drosophila* mediates expression of drosomycin, an antimicrobial peptide that is potently antifungal. It was surmised that, in the absence of the serpin Spn43Ac, Spaetzle is proteolytically cleaved to a dimer, which becomes the extracellular ligand of Toll. Following fungal infection, an unidentified pattern recognition receptor initiates a cascade of serine proteases that leads to the cleavage of Spaetzle, activation of Toll and transcriptional upregulation of the drosomycin gene. In adult flies, phosphorylation of Cactus by an as yet unknown kinase releases the Rel transcription factor Dif, which translocates into the nucleus and activates

certain genes, including those encoding antifungal peptides (Hoffmann *et al.*, 1999; Levashina *et al.*, 1999; Christophides *et al.*, 2002; reviews).

Examination of the genome sequences of ***Anopheles gambiae*** showed that the intracellular components of the Toll and Imd pathways are present in this mosquito, although the Toll pathway is more complex than that of *Drosophila*. *Anopheles gambiae* has 11 *Toll* genes, of which only four (*Toll6*, *7*, *8* and *9*) are unambiguous orthologues of their *Drosophila* counterparts. Gene reduplication generated four *Anopheles* genes (*Toll1A*, *1B*, *5A*, *5B*), which form an orthologous group with two *Drosophila* genes, and single genes encoding orthologues of Cactus, MyD88, Tube and Pelle have been identified (Christophides *et al.*, 2002).

From those of the nearest common ancestor, the *Toll* genes of culicids and drosophilids evolved through independent gene duplication and sequence divergence. Differences between those families are seen in the absence from *An. gambiae* of an orthologue of the *Drosophila* Toll-associated extracellular receptor and of the transcription factor Dif. Although the Toll and Imd signal transduction pathways are known to play key roles in defences against pathogens and parasites, the precise mechanisms and roles of those pathways in defence of *An. gambiae* are unclear (Garver *et al.*, 2008).

42.5.2 Transcription factors

Transcription of immune genes is effected by the binding of transcription factors to regulatory elements on the genes. Mammals and insects share conserved pathways in which transcription factors of the NF-κB class are principal regulators. These nuclear factors (NFs) are members of the Rel protein family, and so share the highly conserved 'Rel homology domain' of 300 amino acids, situated towards the N-terminus. They regulate the expression of immune and other genes by forming homo- and heterodimers that bind to DNA regulatory sites and trigger the expression of specific genes. In mammals, the most common dimers are members of the NF-κB class. They are normally retained in the cytoplasm in inactive form through association with IκB-α, a member of the IκB class of Rel proteins. A processed immune signal dissociates NF-κBs from IκB-α, enabling them to enter the nucleus and activate gene expression (Tomida and Tsuruo, 2002).

(a) Characteristics of transcription factors in Drosophila melanogaster

Insects possess NF-κB-like transcription factors and their I-κ-B-like inhibitors, which are orthologous with those in mammals. The best known are those of *D. melanogaster*, i.e. three NF-κB-like factors named Dorsal, Dif and Relish, and the I-κB-like factor named Cactus, an inhibitor specific to the Toll immune pathway. All affect the expression of immune genes, but Dorsal is also a key regulator of embryonic dorsolateral polarity. Dorsal, Dif and Relish are involved in two intracellular signalling pathways – Toll and Imd. In their naïve state, Dorsal and Dif are retained in inactive form in the cytoplasm of fat body cells by the inhibitory protein Cactus. As noted above, activation of the Toll receptor leads to the degradation of Cactus, when Dorsal or Dif translocates to the nucleus, where it activates the transcription of target genes (Shin *et al.*, 2006).

(b) Characteristics of transcription factors in mosquitoes

The NF-κB transcription factors and their inhibitors produced by *St. aegypti* and *An. gambiae* are orthologous with those of *D. melanogaster*, with the exception of Dif, which arose by a recent duplication of Dorsal in the *Drosophila* lineage (Table 42.1).

The immune responses of ***Stegomyia aegypti*** have been investigated in relation to attacks by the fungus *Beauveria bassiana* and by $Gram^+$ and $Gram^-$ bacteria. The fungal-specific response involved the Toll signal transduction pathway through AaRel1. Alternative transcripts of *AaRel1* encoded two isoforms, AaRel1-A and AaRel1-B, which bind to different κB motifs from immune-gene promoters. Knockout of *AaRel1* showed AaRel1 to be a key regulator of the Toll pathway antifungal response, acting via the ligand Spaetzle (Spz) to link extracellular signals to the Toll

intracellular transduction pathway. Challenge by *B. bassiana* induced in *St. aegypti* five proteins (Toll1A, 1B, 4B, 5A, 5B) that were homologues of the *Drosophila* Toll receptor, and three (Spz1A, 1B, 1C) that were homologues of its ligand Spaetzle. Three of the receptor variants and one homologue of Spz were specifically induced in the mosquito fat body. Knockout experiments suggested that, in *St. aegypti*, Toll5A and Spz1C function as cytokine receptor systems specific to the Toll-receptor mediated immune response (Shin *et al.*, 2006). Further experimental evidence revealed a response mediated by AaRel2 against infection by Gram$^-$ bacteria (*E. coli*, *Escherichia cloacae*, *Pseudomonas aeruginosa*); three Gram$^+$ bacteria were little affected. AaRel2 specifically binds to κB motifs in promoters of defensin, and is known to be an important regulator of the Imd pathway (Shin *et al.*, 2003, 2005; Bian *et al.*, 2005).

In response to bacterial infection of ***Anopheles gambiae***, Rel1 of the Toll pathway was activated, being translocated from the cytoplasm to the nucleus of fat body cells. On the Imd pathway, alternative splicing of the gene *REL2* gave rise to two isoforms, Rel2-F, a full-length isoform, and Rel2-S, which is shorter and lacks the C-terminal inhibitory ANK (ankyrin repeat) and death domains. *REL-F* and *REL-S* transcripts were expressed constitutively in *An. gambiae* during the larval and adult stages. Of the two, *REL-F* was expressed more strongly. Rel2-F functioned to limit infections of Gram$^+$ bacteria and of *Plasmodium*, whereas Rel2-S limited infections of Gram$^-$ bacteria (Meister *et al.*, 2005).

Structurally, the gene *REL2* of *An. gambiae* includes a region that encodes proteins implicated in protein–protein interactions, followed by a Rel homology domain (RHD), a DNA-binding domain, ANK and death domains, and a nuclear localization signal. The gene *TEP1*, which is activated by Rel1 and Rel2, contains in its promoter region sequence motifs similar to the canonical NF-κB binding sites (Frolet *et al.*, 2006). Rel2 regulates expression of the antimicrobial genes *CEC1*, *CEC3* and *GAM1* (Barillas-Mury *et al.*, 1996). On the strength of their activities against the bacteria *Staphyloccus aureus* (Gram$^+$), *M. luteus* (Gram$^-$), and *Plasmodium berghei*, Meister *et al.* (2005) concluded that the Rel2-F isoform functions in defence against Gram$^+$ bacteria and *Plasmodium*, whereas Rel2-S functions in defence against Gram$^-$ bacteria.

42.6 ANTIMICROBIAL PEPTIDES

Infection of insects by bacteria, fungi or protozoans induces the expression of small proteins that show antimicrobial activity *in vitro*. Much work has been carried out on their transcriptional and translational regulation, but very little is known about their mode of action *in vivo*. Antimicrobial peptides are produced in the fat body, haemocytes and certain other tissues, and their induction and release into the haemolymph are an important part of the humoral immune response.

The isolation of genes with immune functions has relied in part on identifying genes that are transcriptionally upregulated after the inoculation of bacteria. Typically, adult mosquitoes are inoculated with a combination of the Gram$^-$ *Escherichia coli* and Gram$^+$ *Micrococcus luteus*, and at intervals after inoculation mRNAs are analysed. Many of the immune-inducible genes reached peak mRNA abundance within the first 24 h after inoculation, and then returned to pre-inoculation levels. Exceptions were found in the continued presence of transcripts of a *defensin* gene, as described below.

Usually, antimicrobial peptides are absent from non-challenged insects, but they are synthesized very soon after immune stimulus and enter the haemolymph, where their concentrations range between 1 and 10 μM. After experimental challenge with live or heat-killed pathogens, antimicrobial activity persists in the haemolymph for one to several days. This response shows no memory, and similar responses are induced by different microorganisms. The positively charged antimicrobial peptides are attracted to the negatively charged surface of bacteria, and most are thought to cause lethal damage to bacterial membranes. Three classes of antimicrobial peptide have been found in mosquitoes: defensins, cecropins and gambicin (Lowenberger, 2001; Christophides *et al.*, 2002; Levashina, 2004).

Defensins are cationic peptides of some 33 to

46 amino acids. They are characterized by six highly conserved cysteine residues arranged in three intramolecular disulphide bridges. Their secondary structure is of an N-terminal loop, an α-helix and two twisting antiparallel β sheets (Hetru *et al.*, 1998). Defensins are widely distributed among insects, and are mainly active against Gram$^+$ bacteria, killing relatively slowly; however, some Gram$^-$ bacteria are sensitive to them (Lowenberger *et al.*, 1995). A defensin gene has been identified in *Culex pipiens* (Bartholomay *et al.*, 2003). Three defensin isoforms were found in adult *St. aegypti* and named A, B and C; however, A and B are so similar that they are thought to be allelic variants of one gene. Defensin was not detectable in naïve *St. aegypti*, and first becomes detectable several hours after bacterial challenge. Its concentration in the haemolymph at 24 h post-challenge was 45 μM. Transcripts for defensin C were found in the midguts of naïve adult *St. aegypti*, whereas isoforms A/B were transformed in the fat body after immune activation (Lowenberger *et al.*, 1995, 1999b; Lowenberger, 2001).

Following inoculation with bacteria, a defensin was isolated from larvae and adults of *An. gambiae*; in the pupae, defensin mRNA was expressed constitutively (Richman *et al.*, 1996); it was later named *An. gambiae* defensin 1, and its gene *AgDef1*. An additional three putative *defensin* genes were identified in the *An. gambiae* genome by Christophides *et al.* (2002). In females orally infected with *P. berghei*, *AgDef1* mRNA was upregulated locally in the midgut at 24 h after blood feeding, and also in the thorax (taken to be in the fat body). Later the mRNA was upregulated in the salivary glands at times coinciding with parasite penetration of the salivary-gland epithelia (Dimopoulos *et al.*, 1998).

After immune challenge with certain Gram$^+$ species of bacteria, haemolymph perfused from *An. gambiae* contained 0.4–0.75 μM defensin; in contrast, females challenged with two strains of the Gram$^-$ *E. coli* contained much higher concentrations. Recombinant *An. gambiae* defensin was active against most Gram-positive bacteria tested, killing them within one minute at concentrations of 0.1–0.75 μM (Vizioli *et al.*, 2001b). Adult female *An. gambiae* inoculated with a single species of bacterium showed a moderate sensitivity to *S. aureus* (Gram$^+$) and a slightly lower sensitivity to *E. coli* (Gram$^-$). *In vivo* knockout of the *defensin 1* gene with dsRNA markedly increased the sensitivity to *S. aureus* and moderately increased that to *E. coli* (Blandin *et al.*, 2002). At 1 and 24 h after bacterial challenge, both defensin and phenol oxidase were present in melanized capsules enclosing bacteria. Doubt was thrown on the idea that defensin has a role in the production or function of melanized capsules by the knowledge that it was present at high titre in the haemolymph of infected mosquitoes and could be randomly incorporated (Hillyer and Christensen, 2005).

Exceptions to the usual finding that immune-inducible genes reach peak mRNA abundance within the first 24 h after inoculation with bacteria and then return to pre-inoculation levels were found in the persistence of transcripts of the *defensin* gene in two species. After inoculation with a suspension of *E. coli* and *M. luteus*, transcripts remained abundant for at least 12 days in the bodies of *An. gambiae* (Gorman and Paskewitz, 2000; citing unpublished data) and clearly detectable for at least 21 days in *St. aegypti* haemolymph (Lowenberger *et al.*, 1999b). Light was thrown on these findings by measurements of the persistence of bacteria after inoculation of single species into *An. gambiae*. The species *M. luteus* was not detected at the first test after inoculation, whereas *E. coli* remained at the highest density on day 12 post-inoculation. The rapid clearance of *M. luteus* showed that it could not be a persistent inducer of defensin. In contrast, the long-term upregulation of *defensin* genes could be explained by *E. coli* acting as a continuous immune elicitor (Gorman and Paskewitz, 2000).

Defensin was strongly induced in the midgut of *An. gambiae* 20–30 h after infection with *P. berghei*, a period during which ookinetes traverse the midgut epithelium. At 10 and 25 days post-infection, defensin was not present in the midgut but was present in the salivary glands (Richman *et al.*, 1996; Dimopoulos *et al.*, 1997, 1998). However, knockout of *defensin* genes with dsRNA had no

significant effect on the viability of *An. gambiae* infected with *P. berghei*, and no effect on oocyst numbers (Blandin *et al.*, 2002).

Cecropins are small, helical peptides with a molecular mass of 2–4 kDa. They lack cysteine residues, and consist of two α-helices linked by a short hinge. The N-terminal region is an amphipathic α-helix with hydrophobic and hydrophilic parts. It includes a high proportion of basic amino acids, which confer a net positive charge. The C-terminal half includes a long stretch of hydrophobic residues. Unlike most insect cecropins, those from mosquitoes lack a tryptophan at position 2, and may not have a C-terminal amidation (Hultmark, 1993; Hetru *et al.*, 1998). Cecropins are active at micromolar concentrations against $Gram^+$ and $Gram^-$ bacteria, targeting the microbial membrane, and can cause lysis within a minute. Most eukaryotic cells are resistant to them.

These peptides are now distinguished by numerals rather than letters, e.g. cecropin1 rather than cecropin A. Four *cecropin* genes were identified in the *An. gambiae* genome by Christophides *et al.* (2002), and three, *cecropin1 2* and *3*, were found to form a cluster in that species by Zheng and Zheng (2002). The two peptides cecropin1 and cecropin3 were reported (as A and C) from *Cx. pipiens* (Bartholomay *et al.*, 2003). A cecropin molecule of 34 amino acids was isolated from the haemolymph of adult *St. aegypti* after bacterial challenge. It was active against certain Gram-negative bacteria at 0.25–2.5 μM and against certain fungi at 1–10 μM (Lowenberger *et al.*, 1999a).

The genes *cecropin1–3* of *An. gambiae* could be regulated by transcription factors of the NF-κB family, which were upregulated by lipopolysaccharides (Zheng and Zheng, 2002). Cecroprin expression could be induced in adult *An. gambiae* by inoculation with bacteria or feeding on blood infected with *Plasmodium berghei*. The cecropin cDNA (complementary DNA) encoded a peptide of 58 amino acids, with a glycine residue at the C-terminus. Among the pre-adult stages, only in young pupae could cecropin expression be induced (Vizioli *et al.*, 2000). *Cecropin2* was upregulated during salivary gland invasion by sporozoites (Rosinski-Chupin *et al.*, 2007).

Gambicin appears to be specific to mosquitoes. The *An. gambiae* genome contains the gene *GAM1* (Christophides *et al.*, 2002), also termed *gambicin*. In that species, gambicin is secreted as a 61-amino acid, mature peptide containing eight cysteines engaged in four disulphide bridges. Its molecular mass is ~6.8 kDa (Vizioli *et al.*, 2001a). In *Cx. pipiens* the *gambicin* gene encodes a deduced 85-amino acid precursor peptide containing a 22-amino acid prepropeptide. Within the sequence are eight conserved cysteine residues surmised to be involved in disulphide bridges (Bartholomay *et al.*, 2003).

Gambicin is induced in the anterior midgut and fat body of adult *An. gambiae* following natural infection with bacteria or *P. berghei*. In bioassays it showed higher potency against the $Gram^-$ *M. luteus* than the $Gram^+$ *E. coli*. Knockout of *gambicin* resulted in an increase in viable oocysts in females infected with *P. berghei*, suggesting that the ookinetes are susceptible to gambicin (Vizioli *et al.*, 2001a).

The induction of antimicrobial agents in mosquitoes has been investigated in adult mosquitoes inoculated with bacteria or naturally infected with malaria parasites. Genes of *An. gambiae* that regulated anti-*Plasmodium* activity also influenced the mosquito's resistance to bacterial infection; but, in contrast, several genes with an effect on resistance to bacterial infection did not affect *Plasmodium* survival. Dong *et al.* (2006) considered that, in nature, bacteria and fungi are the pathogens to which mosquitoes are principally exposed, and surmised that it was unlikely that mosquitoes had evolved specific anti-*Plasmodium* defence mechanisms.

42.7 HAEMOCYTE TYPES AND THEIR CHARACTERISTICS

Descriptions have been recorded of the characteristics of cells present in the haemolymph of 1–4-day-old adult female *Stegomyia aegypti* and *Armigeres subalbatus* (Hillyer and Christensen, 2002; Hillyer *et al.*, 2003b). The cells were collected by volume displacement (perfusion) of haemolymph with saline, and examined by light- or electron

microscopy and by immunocytochemistry. The same four distinct cell types were identified in haemolymph from the two species. That was fewer than the number of haemocyte types that had been described from mosquitoes by earlier authors (Volume 1, Section 9.3.2) because a number of types were now classified together as granulocytes (Table 42.2). Two cell types in the perfusates were haemocytes that are normally present in circulating haemolymph, i.e. granulocytes and oenocytoids. The other two cell types, adipohaemocytes and thrombocytoids, are not normally present in circulating haemolymph, and their presence in the perfusates was thought to be an experimental artefact. The following descriptions are of the cell types recovered from *St. aegypti*. The homologous cells recovered from *Ar. subalbatus* were structurally and cytochemically very similar.

Granulocytes were the most abundant cell type in perfused haemolymph. These cells are of about 9 µm diameter, the nuclei contain both euchromatin and heterochromatin, and no prominent nucleolus is visible. The cytoplasm is rich in polyribosomes. The granulocytes were shown to be polymorphic, the ultrastructure of their cytoplasm revealing three subgroups: (i) small cells containing few granules but rich in rough endoplasmic reticulum (RER); (ii) cells of normal size, rich in RER and containing membrane-bounded vesicles in moderate numbers; and (iii) cells of normal size containing little or no RER, the cytoplasm filled with membrane-bounded vesicles. Four types of membrane-bounded vesicles were distinguished, each granulocyte possessing one or more types: (i) structured electron-lucent granules; (ii) structureless electron-dense granules; (iii) structured electron-dense granules forming

Table 42.2 Characteristics of the circulating haemocytes and of non-circulating cell types present in haemolymph that had been obtained by perfusion from 1–4-day-old adult female *Stegomyia aegypti*. (From the data of Hillyer and Christensen, 2002, 2005; Johnson *et al.*, 2003.)

Type	*Earlier names – where different*	*Structural characteristics*	*Enzymes or enzyme activities present (cited names)*	*Abundance*
Circulating				
Granulocytes	Prohaemocytes Granular haemocytes Plasmatocytes Coagulocytes	Polymorphic; 9 µm diam. No prominent nucleolus. Cytoplasm rich in ribosomes; in most cells with granules having lysosomal activity.	Acid phosphatase Alpha-naphthyl acetate esterase[1] Phenylalanine hydroxylase[2]	Abundant
Oenocytoids	–	Spherical; 9 µm diam. Nucleus eccentric, nucleolus prominent. Mitochondria few.	Phenol oxidase[3] Peroxidase (in some cells) Phenylalanine hydroxylase[2]	Few
Non-circulating				
Adipohaemocytes	Spherulocytes	Ovoid cells, length 40 µm. Indistinguishable from fat body cells. Presence probably an artefact of perfusion.	–	Common
Thrombocytoids	Nephrocytes Cystocytes	Irregularly shaped cells; c. 30 µm diam. Large nucleus; homogeneous cytoplasm. Probably not circulating cells but normally attached to fixed tissues.	–	Rare

IUMB (International Union of Biochemistry and Molecular Biology) accepted names: [1], Carboxylesterase; [2], Phenylalanine 4-monooxygenase; [3], Monophenol monooxygenase.

myelin-like figures; and (iv) structureless electron-lucent granules. Evidence of three enzymes has been reported: acid phosphatase, which is present in most granules, indicating their lysosomal nature; alpha-naphthyl acetate esterase, indicating non-specific esterase activity; and phenylalanine hydroxylase, present mostly in the nucleus, as reported by Johnson *et al.* (2003). Of the two types of circulating haemocyte, granulocytes are active in phagocytosis of microorganisms, in structural aspects of encapsulation of nematodes' microorganisms and microfilariae, and in wound healing (Sections 42.8, 42.9, 42.10.2).

The **oenocytoids** are spherical cells of approximately 9 μm diameter. They are few in number, and are characterized by having an eccentric nucleus, no prominent nucleolus and a homogeneous cytoplasm. The only visible organelles are a few poorly developed mitochondria and lysosomes. Polyribosomes are scattered throughout the cytoplasm. Large crystalline-like bodies can occasionally be seen in the cytoplasm. The presence of three enzymes that function in immune responses, including encapsulation, has been reported: phenol oxidase, in foci scattered throughout the cytoplasm; peroxidase, in the cytoplasm of some but not all oenocytoids; and phenylalanine hydroxylase, reported by Johnson *et al.* (2003) in the nucleus and scattered through the cytoplasm. The formal names and EC numbers of these enzymes are given in Table 42.3. Earlier it had been found that pro-phenol oxidase is constitutively in the haemocytes of *Ar. subalbatus* mosquitoes, but not in the fat body, ovaries or midgut. Even so, phenol oxidase has been found throughout the mosquito body (Huang *et al.*, 2001).

Cells in perfused haemolymph that had the characteristics of **adipohaemocytes** were considered indistinguishable from fat body cells (trophocytes). These cells, of about 40 μm diameter, were the second most common cell type. They contained a large nucleus with a well-developed nucleolus. The cytoplasm contained large lipid droplets, mitochondria adjacent to membranes, a few electron-dense granules, and many glycogen particles. It was thought likely that these were not circulating blood cells but artefacts of the isolation process. Similarly, the rarely seen **thrombocytoids** were thought normally to be fixed to tissues, but freed and suspended in haemolymph by perfusion. These were irregularly shaped cells of 30 μm diameter; the cytoplasm was homogeneous with poorly developed organelles, and showed many cytoplasmic invaginations. All four cell types could be distinguished from one another by the lectins that bound to them.

42.8 PHAGOCYTOSIS

42.8.1 Phagocytosis of bacteria

To examine the responses of mosquitoes to invasive bacteria, a steel probe of 0.15 mm diameter was dipped into a pellet of centrifuged bacteria and briefly inserted through the neck membrane. After different time intervals, haemolymph was removed by perfusion and fixed in glutaraldehyde for examination by electron microscopy (Hillyer *et al.*, 2003a). The primary responses occurred in the haemocoel and were, depending on bacterial species, principally phagocytosis by circulating haemocytes or principally haemocyte-mediated encapsulation. When batches of *St. aegypti* were inoculated with one or another of four species of $Gram^+$ bacteria and four species of $Gram^-$ bacteria, in each case the immune response was mediated by haemocytes and was either predominantly phagocytosis or predominantly encapsulation. Overall, there was no general effect of Gram type; however, the primary response of *St. aegypti* in clearing *Escherichia coli* ($Gram^-$) was phagocytosis, only a small proportion being encapsulated, whereas most *Micrococcus luteus* ($Gram^+$) were encapsulated, with some encapsulated bacteria later being phagocytosed. Phagocytosis of unencapsulated *M. luteus* was infrequent.

Not surprisingly, the eight bacterial species used in those experiments differed in pathogenicity. Taking the two extreme cases, by 24 h even the highest dose (200×10^3) of *Staphylococcus aureus* had caused only trivial mortality. With *Bacillus cereus*, in contrast, survival was negligible 3 h after inoculation of the 200×10^3 dose, and after 6 h there was no survival from the 20×10^3 dose

(Hillyer *et al.*, 2004). The higher inoculated loads experienced by these mosquitoes were unnaturally large. Probably, when mosquitoes are subject to a natural bacterial challenge, as from a small wound, they can counter bacterial invasion by phagocytosis or encapsulation and clear any infection rapidly. However, that is not the case with mosquitoes that are susceptible and subject to oral infection with *Bacillus thuringiensis* or *Bacillus sphaericus*. In such cases bacterial endotoxins bind to receptors on the midgut epithelium, leading to pore formation, the destruction of midgut cells, invasion of other internal organs and death (Sections 46.5.7, 46.6.5).

The fullest descriptions of phagocytosis are from experimental studies with *St. aegypti* (Hillyer *et al.*, 2003a, 2004, 2005), on which the account below is based, but the findings from *Ar. subalbatus* as host were very similar (Hillyer *et al.*, 2003b). Bacteria were inoculated into adult female *St. aegypti*, usually in doses of 2, 20 or 200 × 10^3 bacteria. Phagocytosis was predominantly by granulocytes, but very occasionally oenocytoids were seen to phagocytize bacteria. Inoculation of bacteria expressing GFP revealed that phagocytosis started within 5 min; over time some granulocytes phagocytized >100 bacteria. (Later ultrastructural studies indicated a tenfold greater phagocytic capability.) Strong fluorescence from bacteria was observed after 3 h, but after 24 h the signal had almost completely disappeared, indicating bacterial degradation. In most cases, the bacteria became enclosed in phagosomes, membrane-bounded vesicles formed by inward-folding of the cell membrane. Three forms of internal structure were seen: (i) tight phagosomes, in which the vesicle membrane surrounded an individual bacterium tightly; (ii) loose phagosomes, in which the vesicle membrane surrounded an individual bacterium loosely, and in which the bacterium had been encapsulated or was surrounded by flocculent material; and (iii) large vesicles containing a number of bacteria.

42.8.2 Phagocytosis of a yeast

Cultured cells of the yeast *Candida albicans* (Saccharomycetales) that were inoculated into adult female *Culex quinquefasciatus* were progressively cleared from the haemolymph. From a mean of 972 yeast cells/insect recovered immediately after inoculation, the number had declined by 66% after 6 h and by 98% after 24 h. The numbers of haemocytes also changed during that 24 h period. In untreated females, the estimated mean number was 195.4 (± 22 s.d.), comprising oenocytoids (7.2%) and granulocytes (92.8%) (the latter including 38% of cells identified as prohaemocytes and plasmatocytes). The total mean haemocyte count increased to 375/mosquito at 6 h post-inoculation, was still greater than the control at 18 h, and then decreased. At 6 h post-inoculation, granulocytes were involved in phagocytosis, and up to six yeast cells were found in a single granulocyte. At 18–24 h post-inoculation, surviving yeast cells became enclosed in pigmented capsules of varying wall thickness (Da Silva *et al.*, 2000).

42.8.3 Phagocytosis of *Plasmodium*

Stegomyia aegypti is susceptible to infection with *Plasmodium gallinaceum*, an experimental host/parasite combination. After oral infection, development to the oocyst stage and release of sporozoites into the haemocoel, sporozoites were phagocytosed and processed inside membrane-bounded vesicles (Hillyer *et al.*, 2003a). Only about 20% of oocyst sporozoites enter the host's salivary glands, but the contribution of phagocytosis to the destruction of sporozoites is small (Volume 4, Section 51.9). The contribution of phagocytosis was measured in haemolymph perfusates from infected females. During periods when sporozoites of *P. berghei* were circulating in the haemocoel of *An. gambiae*, only 3.5% of haemocytes contained sporozoites. Of those, 96% contained only one sporozoite and 4% contained two (Hillyer *et al.*, 2007).

42.9 STRUCTURAL OBSERVATIONS OF ENCAPSULATION

In many arthropods, one form of immune response to invasive organisms is encapsulation, enclosure within a proteinaceous capsule. In insects generally,

haemocytes constitute an important part of the capsule, but in culicids and certain other nematoceran Diptera, depending on the parasite, the capsules may not include haemocytes. The terms 'cellular encapsulation' and 'acellular encapsulation', respectively, would loosely describe the two processes. The response of mosquitoes to bacteria and malaria parasites is acellular encapsulation, whereas that to nematodes is cellular encapsulation. In mosquitoes, encapsulation occurs mostly within the haemocoel or under the basal lamina surrounding the midgut, where haemolymph proteins can penetrate. However, onchocercid nematodes that have penetrated host flight muscles and developed to larvae become encapsulated.

In outline, studies of innate immune processes in mosquitoes indicate that foreign organisms that enter the haemocoel are quickly identified as non-self, that 'recognition proteins' bind to them, and that a number of effector processes are activated, including encapsulation. Concurrently, pro-phenol oxidases (zymogens) in the haemolymph are activated, triggering an enzymatic cascade that leads to the inclusion of melanin in the capsules.

Brief descriptions are provided here of the encapsulation by mosquitoes of bacteria, malaria parasites and nematodes.

42.9.1 Encapsulation of bacteria

When bacteria were inoculated into adult mosquitoes, immune responses involving haemocytes occurred in the haemocoel, taking the form of either mostly phagocytosis or mostly encapsulation, depending on the bacterial species. For example, the immune response of *St. aegypti* and *Ar. subalbatus* to *Micrococcus luteus* was predominantly encapsulation, whereas that to *E. coli* was predominantly phagocytosis (Hillyer *et al.*, 2003b, 2004).

When *Armigeres subalbatus* were inoculated with *E. coli*, encapsulation started as soon as 5 min post-inoculation, but over time only a relatively small proportion of the bacteria were encapsulated, most were phagocytosed. At 1 h post-inoculation some *E. coli* were enclosed in well-developed and pigmented capsules, and some of these capsules had been phagocytosed by granulocytes. Most *M. luteus* inoculated into *Ar. subalbatus* became encapsulated. Light microscopy of living haemocytes in perfused haemolymph showed that within 5 min of inoculation some of the bacteria were enclosed in black or dark brown capsules. At 1 h post-inoculation, heavily pigmented capsules of 2 μm diam. were observed. Immunocytochemistry of haemolymph samples taken at 0.5 and 24 h post-inoculation revealed the presence of phenol oxidase within and around capsules surrounding *M. luteus* and *E. coli*; it was most frequently observed in capsules in the initial stages of pigmentation. Electron microscopy showed the capsules to be unsheathed and composed of many granules. The granules surrounding *E. coli* in *St. aegypti* were of medium electron density; in contrast, the granules surrounding *M. luteus* in *Ar. subalbatus* were of high electron density, and many of those capsules became impenetrable to embedding resin (Hillyer *et al.*, 2003a,b; Hillyer and Christensen, 2005).

At 24 h after *E. coli* or *M. luteus* had been inoculated into *St. aegypti*, phenol oxidase was present in melanized capsules surrounding the bacteria, and was detected also bound to bacteria that had been phagocytosed by granulocytes. Because phenol oxidase is produced by oenocytoids and not by granulocytes, it was proposed that the enzyme binds to bacteria in the haemolymph, where it initiates melanization prior to phagocytosis (Hillyer and Christensen, 2005). Previously, granulocytes of *Ar. subalbatus* had been reported to phagocytize bacteria enclosed in melanized capsules (Hillyer *et al.*, 2003b).

42.9.2 Encapsulation of malaria parasites

During the development of *Plasmodium* within an adult mosquito, ookinetes migrate across the midgut epithelium to a position between the outer cell-surface of the epithelium and the surrounding basal lamina, and it is in that position that they are first exposed to immune factors in the haemolymph; apparently its soluble constituents

can cross the basal lamina. No involvement of haemocytes can be seen. The recognition protein TEP1, produced by haemocytes, becomes bound to the surface of ookinetes when they first emerge from the epithelial cells into the surrounding extracellular space enclosed by the basal lamina; in some cases TEP1 is bound to early-stage oocysts in the same location (Blandin *et al.*, 2004).

Ultrastructural studies were conducted on susceptible and refractory lines of *An. gambiae* infected with the simian parasite *Plasmodium cynomolgi*. In females of the susceptible line, the ookinetes and oocysts developed normally, whereas few parasites infecting the refractory line survived beyond the ookinete or early-oocyst stage. Ookinetes infecting refractory females became coated with electron-dense particles when they escaped from the epithelial cells and entered the extracellular space enclosed by the basal lamina. In fixed preparations, these ookinetes often seemed to be embedded in a sparse 'matrix'. Phenol oxidase was present in cells of the midgut epithelium. Incubation of that tissue with DOPA or dopamine led to the deposition of electron-dense particles in the basal labyrinth of the midgut cells, in the basal lamina near encapsulated ookinetes, or in the capsule itself. Particles appeared also on the haemocoel side of the basal lamina, adjacent to developing capsules, thickening the capsule wall over that area. The wall of mature capsules was 5–10 μm thick and highly electron dense, so that in micrograph sections of capsules it appeared black (Paskewitz *et al.*, 1988, 1989).

In refractory females of *An. gambiae* infected with *P. berghei*, membrane-bounded 'actin zones' containing filamentous actin were often seen surrounding partly lysed and dying ookinetes within the basal extracellular space beneath the basal lamina. By 48 h post-infection, 99.5% of ookinetes were dead, surrounded by a zone of polymerized actin which was formed in adjacent, healthy epithelial cells. Knockout of *TEP1* blocked formation of actin zones and enabled most ookinetes to survive to the oocyst stage; however, some ookinetes were lysed independently of TEP1 (Shiao *et al.*, 2006).

Evidence from the field suggests that encapsulation of ookinetes occurs much less frequently in infected wild mosquitoes than in refractory colonized mosquitoes (Brey, 1999; Schwartz and Koella, 2002).

42.9.3 Encapsulation of nematodes

(a) Mermithid nematodes

Preparasitic juveniles of *Romanomermis culicivorax* invaded *Culex territans* larvae successfully, but electron microscopy revealed that between 12 and 24 h after entry a thin homogeneous deposit covered each parasite. Shortly after this, granulocytes became numerous and started to adhere to the deposit. Between about 60 and 84 h the granulocytes formed a thick clear capsule enclosing the parasite. During this period the parasites became moribund, and by the fourth day all were dead. On the fourth and fifth days the parasites started to disintegrate and were, seemingly, being digested. During the same period most of the surrounding blood cells became necrotic and started to lose their integrity. After 5 days, when the parasites had begun to disintegrate, the capsule wall consisted mainly of cellular debris with small pigmented areas. The capsules were never completely pigmented. Eventually, both capsule and parasite disappeared (Poinar *et al.*, 1979).

(b) Onchocercid nematodes

Encapsulation occurs sequentially in two different locations: (i) extracellular, when microfilariae are in the haemocoel of the host; and (ii) intracellular, after microfilariae have invaded the host organ, flight muscle or Malpighian tubule, in which further development will occur.

When female *Anopheles quadrimaculatus* ingested *Brugia pahangi* microfilariae from infected cats, an immune response was rapidly initiated. Within 20 min microfilariae had penetrated into the haemocoel, and already both sheathed and ex-sheathed microfilariae were coated with a homogeneous layer of electron-dense particles. After 1 h the layer was 0.5–2.5 μm thick, and by 2 h the microfilariae were

enclosed in acellular capsules on which granulocytes had started to settle. By 12–16 h, some acellular capsules were completely surrounded by granulocytes, and a layer with the ultrastructural appearance of a basal lamina had appeared over the outer surface of the granulocytes. This appeared to be secreted by the granulocytes, and once the layer was formed no more granulocytes settled on the capsule. By 24–48 h post-infection the completed capsules consisted of an inner acellular layer and an outer cellular layer which was markedly vacuolated. The enclosed microfilariae were necrotic at this time (Chen and Laurence, 1985; Chen, 1988; Liu *et al.*, 1998).

Intracellular encapsulation of parasites is not common in insects, but microfilariae that have invaded flight-muscle cells differentiate to first-stage larvae, and are again at risk of encapsulation. By 21 h post-infection of *Anopheles atroparvus* with *Brugia pahangi*, or much longer after infection of *An. quadrimaculatus* with *Brugia malayi*, a large number of vesicles have started to surround the parasite, and they are replaced by a flocculent mass 1–3 μm thick and bounded by a membrane, apparently derived from the vesicles. Areas of pigmentation, taken to indicate melanin formation and seen in electron micrographs as electron-dense patches, appeared in the layer of flocculent material, first adjacent to the larva, then extending outwards, and eventually visible as an electron-dense innermost layer of the capsule. Larval development was affected before pigmentation of the capsule, indicating that melanin formation was not the main cause of larval pathogenesis. At no time were haemocytes seen to congregate around the infected muscle fibre (Lehane and Laurence, 1977; Chikilian *et al.*, 1994, 1995).

Dirofilaria immitis that migrated from the haemocoel of *Ochlerotatus sollicitans* to the Malpighian tubules, where they had penetrated into primary cells, were affected either as microfilariae or as first-stage larvae. The first evidence of an immune response was the appearance of very fine granules around the parasite. Later, electron-dense patches appeared on the cuticle of the parasite and increased in area during the following days. Eventually this material either covered the larva or was limited to patches over the mouth, anus and intersegmental boundaries (Nayar and Sauerman, 1975; Bradley and Nayar, 1985).

42.10 IMMUNE AND OTHER ROLES OF MELANIN IN MOSQUITOES

Melanins are present in prokaryotes and most eukaryotes, in which they have a wide variety of roles. They can have protective functions: in microorganisms melanins are highly effective scavengers of free radicals, while in humans they serve as a shield against UV radiation by absorbing the appropriate wavelengths. Some melanins are associated with the virulence of hosts towards pathogenic microbes; others increase microbial virulence by reducing the pathogen's susceptibility to host defence (Nosanchuk and Casadevall, 2006, review). Melanin is a component of some immune responses of insects to pathogens and parasites.

In insects, by far the greatest amount of melanin is produced by the epidermis and secreted into the cuticle without any toxic side effects. The amounts produced in immune responses to infection are trivial in comparison, but undoubtedly important. Assertions that by-products of melanin synthesis exert toxic effects on invading pathogens and parasites should be treated as putative until substantiated.

Certain terms used in connection with melanin production and deposition can be taken to have implications that are not appropriate for particular situations, and their use can be misleading. The following definitions are appropriate for descriptions of insect immune responses.

Melanin (*n.*) Any of the black or brown pigments of high molecular mass formed by the actions of oxidases on phenols or indoles followed by polymerization.

Melanize (*v.*) To infiltrate with melanin; (*adj.*) melanized.

Melanogenesis (*n.*) The formation of melanin by living cells. Alternative: melanin biosynthesis.

Melanotic (*adj.*) Pertaining to the presence, normal or pathological, of melanin.
Melanotic encapsulation (*v.*) The encasement of pathogens or parasites within a capsule containing melanin, with or without immediate haemocyte involvement.

42.10.1 Melanins: structure and characteristics

Melanins are polymers of high molecular mass and varied structure, different types being formed via different biosynthetic pathways. Plant melanins are formed by polymerization of phenolic compounds and are nitrogen free. Most animal melanins are formed by polymerization of indoles and contain nitrogen. In mammals two forms of dermal melanin are produced in melanocytes: the commoner form, eumelanin, is a brown-black polymer; the less common pheomelanin, formed from benzothiazine units, is a red-brown polymer. Eumelanin occurs also in insects, where it functions in innate immune mechanisms.

Insect eumelanins are macromolecules, being polymers derived from 5,6-dihydroxyindole as the monomer (Figure 42.1). Traditionally, melanins have been viewed as extended heteropolymers, but aggregates of smaller oligomeric structures may be a more accurate model. The term 'secondary structure' is used to refer to the supramolecular assembly of monomers. Eumelanins are negatively charged, hydrophobic and unreactive. They are very stable, and have low solubility over a wide range of pH.

Under *in vivo* conditions, melanins bind to a variety of compounds, including proteins. Strictly speaking, the term 'melanin' should encompass both the chromophore and the associated protein, but it has become the norm for the term to refer only to the chromophore. All melanins show broad band absorption of UV and visible wavelengths. With eumelanin, the extended conjugated system of double bonds absorbs all wavelengths from UV through the visible spectrum to the near infrared, so the polymer appears black or dark brown. Eumelanin has powerful antioxidant and free-radical scavenging ability (Tran *et al.*, 2006; Meredith *et al.*, 2008). Melanins have been very difficult to identify and analyse because of their insolubility and heterogeneity, and the lack of methods to split the polymers into their monomeric units. Now melanins can be identified as such by electron paramagnetic resonance spectroscopy (Enochs *et al.*, 1993), and can be characterized by chemical degradation followed by HPLC determination (Wakamatsu and Ito (2002).

42.10.2 Non-immune roles of melanin in mosquitoes

Melanins bond covalently with protein molecules, cross-linking them, and in so doing harden and strengthen proteinaceous structures. A remarkable example of hardening was found in the jaws of the marine polychaete *Glycera dibranchiata*, in which the melanin cross-linking produced exceptional strength (Moses *et al.*, 2006). In insect integument, melanin granules may be produced within epidermal cells and transported to the subepicuticular space, or melanin may be produced within the cuticle matrix, where the covalent bonds form between the polymer and matrix proteins. Probably, covalent links are formed between the polymeric melanin and the matrix proteins, contributing to both darkening and increased mechanical stiffness of the cuticle. In insects generally, sclerotization appears to be a more important mode of cuticle hardening than bonding with melanin (Andersen, 2005). Cuticle hardening has not been investigated in culicids, but it has been surmised that melanin functions in wound healing.

The first response to wounding of the integument of *Ar. subalbatus* larvae was a localized constriction of the wound site. Within 5 min, granulocytes had moved to the wound and clumped together over it, forming a plug, and had lysed, releasing granules. The granules exuded their contents forming a gel-like substance over the wound. This interacted with the plasma to form a coagulum, and a pigmented clot was formed (Lai *et al.*, 2001). Pro-phenol oxidase was present in the granules, and its release was thought to lead to

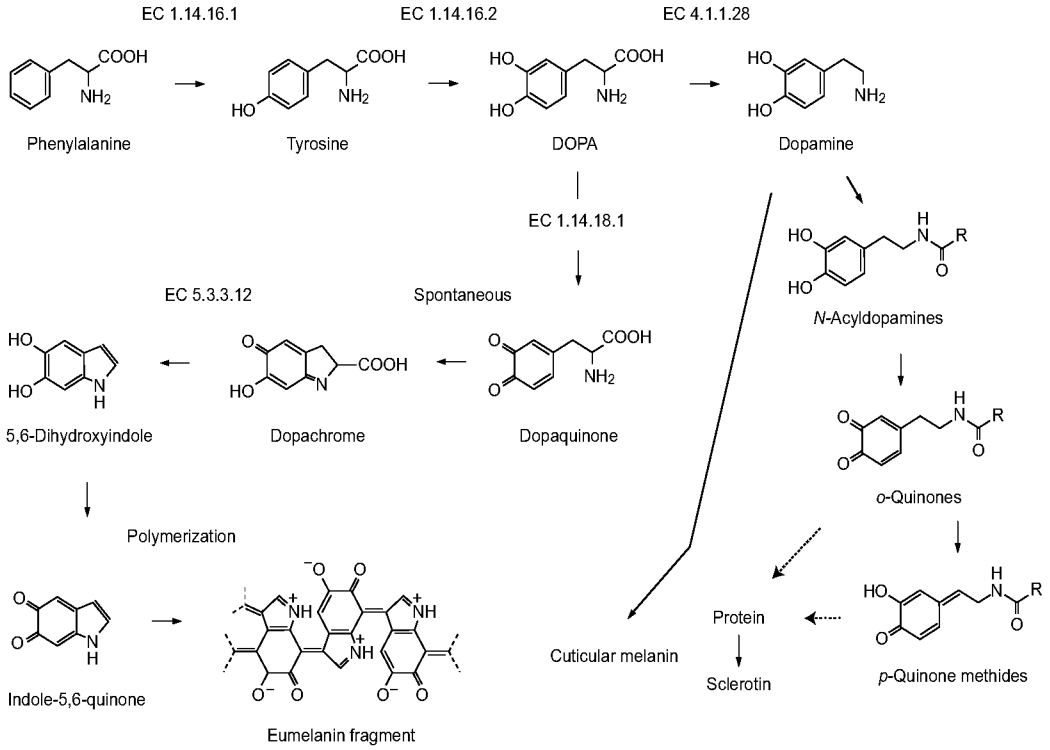

Figure 42.1 Postulated biosynthetic pathways in insects that lead to the formation of eumelanin or to the formation of sclerotizing agents that cross-link proteins and function in the hardening of insect cuticle. (After Sugumaran, 2002; Morgan, 2004; Andersen, 2005; Christensen *et al.*, 2005.) The synthetic steps that follow the formation of 5,6-dihydroxyindole are best known in mammals, and are not shown in detail. The pathway to synthesis of cuticular melanin in insects is not well known. The following enzymes function in the biosynthesis of eumelanin in mosquitoes: EC 1.14.16.1, phenylalanine hydroxylase; EC 1.14.16.2, tyrosine hydroxylase; EC 1.14.18.1, phenol oxidase; EC 4.1.1.28, DOPA decarboxylase; EC 5.3.3.12, dopachrome conversion enzyme.

melanin formation and melanization of the scab (Lai *et al.*, 2002).

The chorion of recently laid aedine eggs is soft and white, but it soon becomes hardened and black, and capable of withstanding desiccation. The presence of melanin might well account for darkening of the chorion, but evidence has not been obtained for a role in its hardening. Such biochemical evidence as there is, from *St. aegypti*, points to two possible means of chorion hardening: *o*-quinone tanning of chorionic proteins; and cross-linking of chorionic proteins following the formation of dityrosine residues on those proteins by the action of peroxidase (Li and Christensen, 1993; Li, 1994; Li *et al.*, 1996a; Kim *et al.*, 2005).

42.10.3 Melanin biosynthesis

Usually, authors use the informal names of enzymes and often without reference to the EC number or IUBMB-accepted name. In this section also the informal names are sometimes used, but their EC numbers and IUBMB-accepted names are indicated in Table 42.3.

The biosynthetic pathway of melanin synthesis as it functions in the innate immune responses of mosquitoes is only incompletely known, and some published illustrations of the full pathway insert information from mammals to fill the gaps. However, Sugumaran (2002) stated that there are significant differences between mammalian and insect pathways – in the enzymes involved, in the

Table 42.3 IUBMB (International Union of Biochemistry and Molecular Biology) accepted names, EC numbers, and synonyms of enzymes involved in the immune responses of mosquitoes, or the biosynthesis of eumelanin, or that have been detected in mosquito haemocytes. Only frequently used synonyms are cited.

Accepted name	*EC number*	*Synonyms/Other names used in mosquito studies*
Catalase	1.11.1.6	–
Peroxidase	1.11.1.7 *	Horseradish peroxidase
Nitric-oxide synthase	1.14.13.39	NO synthase, NOS
Phenylalanine 4-monooxygenase	1.14.16.1	Phenylalanine hydroxylase
Tyrosine 3-monooxygenase	1.14.16.2	Tyrosine hydroxylase
Monophenol monooxygenase	1.14.18.1	Phenol oxidase, phenolase, tyrosinase, PO
Superoxide dismutase	1.15.1.1	SOD
Carboxylesterase	3.1.1.1	Non-specific carboxylesterase, alpha-naphthyl acetate esterase†
Acid phosphatase	3.1.3.2	–
Aromatic L-amino acid decarboxylase	4.1.1.28	DOPA decarboxylase
L-dopachrome isomerase	5.3.3.12	Dopachrome conversion enzyme, dopachrome conversion factor

*, one of a large family of peroxidases.
†, Visualization by a substrate–diazonium dye reaction using alpha-naphthyl acetate led to the term alpha-naphthyl acetate esterase.

nature of the enzymatic reactions and in the nature of the eumelanin polymers. The involvement of four enzymes in the melanogenic and related pathways of mosquitoes has been established by demonstrating a significant reduction in the deposition of melanin around inoculated parasites or Sephadex beads after knockout of individual enzymes. By their informal names, those enzymes were phenylalanine hydroxylase (EC 1.14.16.1), phenol oxidase (EC 1.14.18.1), dopachrome conversion enzyme (EC 5.3.3.12) and the associated enzyme DOPA decarboxylase (EC 4.1.1.28) (Infanger *et al.*, 2004; Shiao *et al.*, 2006; Paskewitz and Andreev, 2008).

Variably, either L-phenylalanine or L-tyrosine is treated as the initial substrate on the pathway of melanin synthesis. Functionally, tyrosine appears the more likely compound because, as described below, further progress on that pathway requires the availability of tyrosine hydroxylase, which itself requires the activation of pro-phenol oxidase. However, experimental evidence suggests that phenylalanine in the haemolymph may be needed to maintain the necessary titre of tyrosine. When knockout of the phenylalanine hydroxylase (EC 1.14.16.1) gene in *Ar. subalbatus* and *St. aegypti* was followed by inoculation with microfilariae of *Dirofilaria immitis*, a significant reduction was found in the proportions of microfilariae that became enclosed in melanized capsules (Infanger *et al.*, 2004). In contrast, knockout of the phenylalanine hydroxylase gene in *An. gambiae* did not affect the formation of melanized capsules around inoculated Sephadex particles, although Paskewitz and Andreev (2008) conceded that further studies on that species were needed.

Taking phenylalanine as the initial substrate on the pathway of eumelanin biosynthesis (Figure 42.1), its hydroxylation produces tyrosine, and in successive steps tyrosine becomes hydroxylated to DOPA (dihydroxyphenylalanine), and DOPA is oxidized to dopaquinone, which is non-enzymatically converted to dopachrome. This 'spontaneous' transformation to dopachrome involves intramolecular cyclization to leucodopachrome (not shown) followed by partial reoxidation. Then, in a rate-limiting step involving DOPA decarboxylase, dopachrome is converted to 5,6-dihydroxyindole. The further synthetic steps are best known from mammalian studies. By enzymatic catalyses (not shown), 5,6-dihydroxyindole is converted first to its single-electron oxidation product, a semiquinone radical, and then to the monomer indole-5,6-quinone. Polymerization proceeds by oxidative

dehydrogenation, resulting in the formation of eumelanin (Johnson *et al.*, 2001; Fang *et al.*, 2002; Christensen *et al.*, 2005; Huang *et al.*, 2005b; Nappi and Christensen, 2005; Meredith *et al.*, 2008).

The monooxygenases that have the informal names tyrosine hydroxylase, tyrosinase, phenolase or phenol oxidase (PO), and that catalyse the conversion of tyrosine to DOPA or of DOPA to dopaquinone, are present in the haemolymph as inactive pro-phenol oxidases (PPOs). Those zymogens are activated after wounding or invasion by pathogens has induced a serine-protease cascade. Those insults trigger a protease to cleave and activate the zymogen of a serine protease; that enzyme, by limited proteolysis, activates another serine-protease zymogen which, in a final step and again by proteolysis, activates the pro-phenolases. Of the many pro-phenol oxidases that have been described from mosquitoes (Waterhouse *et al.* (2007) reported 18), a high proportion function in developmental physiology. The nine *PPO* coding sequences described in *An. gambiae* exhibit different expression profiles during development and in the various physiological states of adult mosquitoes: *PPO1–4* are mainly expressed during the immature stages, *PPO6* and *9* are predominant in adult mosquitoes, and *PPO2*, *3* and *9* are upregulated after a blood meal (Christensen *et al.*, 2005; Michel and Kafatos, 2005; Nappi and Christensen, 2005).

Pathogens or parasites that invade adult mosquitoes are soon recognized as non-self by recognition proteins, thereby triggering innate immune responses (Section 42.4.2). Among these may be the cascade of reactions that leads to eumelanin synthesis. The cascade is initiated by the activation of pro-phenol oxidase, triggered directly or indirectly upon invasion of a host by pathogens or parasites. This makes phenol oxidase available to catalyse what might be considered the first reaction on the biosynthetic pathway for eumelanin synthesis in insects – the hydroxylation of L-tyrosine (Figure 42.1). Exposure of tyrosine to phenol oxidase converts it to 3,4-dihydroxyphenylalanine, commonly called L-DOPA, and the same enzyme oxidizes DOPA to dopaquinone; the pathway is set for eumelanin production.

The recovery of enzymes from the granulocytes and oenocytoids of *St. aegypti* is detailed in Table 42.2. Enzymes that function in eumelanin synthesis in mosquitoes are produced in certain tissues but may function elsewhere. Thus, phenol oxidase, produced in the oenocytoids of *St. aegypti* was, in *Ar. subalbatus*, present in the pigmented acellular capsules that formed around inoculated bacteria (Johnson *et* al., 2003; Hillyer *et al.*, 2003b). In *Ar. subalbatus*, a high transcriptional level of DOPA decarboxylase in haemocytes correlated well with high enzyme activity in the haemolymph. In females carrying microfilariae of *Dirofilaria immitis*, transcripts of DOPA decarboxylase fell markedly in the ovaries but increased in haemocytes, supposedly owing to competition for resources (Li *et al.*, 1994; Huang *et al.*, 2005a). Johnson *et al.* (2001) reported that in adult *St. aegypti* transcripts of DOPA decarboxylase were most abundant in the fat body. The manner of release of these enzymes and of its control is not known.

It is known from studies on other insects that when DOPA has been formed from tyrosine a potential branch point in biosynthetic pathways is reached. In the first case, DOPA may be oxidized to dopaquinone, taking it on a synthetic pathway to the eumelanin that appears in haemolymph and functions in immune responses. Alternatively, in the second case, the DOPA may be decarboxylated to dopamine, which is a putative substrate on at least two other pathways: (i) a pathway to the synthesis of cuticular melanin; and (ii) a pathway to the formation of sclerotizing agents. In the synthesis of *cuticular melanin*, DOPA is decarboxylated to dopamine, which is converted via dopaminechrome to 5,6-dihydroxyindole. In cuticular structures, some melanin is present in granules that are produced by epidermal cells and transported via fine cellular projections to the sub-epicuticular space; there the melanin is linked to granular proteins. Melanin may also be formed within the cuticular matrix, in which it is diffusely distributed and probably is covalently linked to the matrix proteins. In the synthesis of *sclerotizing agents*, dopamine can be acylated to *N*-acyldopamines, notably through catalysis by N-acetyltransferase to *N*-acetyldopamine, or to

N-β-alanyldopamine. Enzymatic oxidation converts both compounds to the respective *o*-quinones, which may isomerize to *p*-quinone methides. Both the *o*-quinones and *p*-quinone methides bond covalently with terminal amino and other groups in protein molecules, cross-linking them, and are putative sclerotizing agents (Andersen, 2005).

42.10.4 Putative roles of melanin in mosquito immune responses

It is appropriate here to cite comments of Nappi and Christensen (2005), made after the extensive research undertaken by different investigations into the role of melanin in the immune processes of insects: 'Experimental evidence defining the role of melanin and its precursors in insect innate immunity is severely lacking'; and again 'there is currently little experimental evidence to accurately define the role of melanin and its precursors in insect innate immunity'.

A conspicuous response of mosquitoes to invasion by most parasites is encapsulation, almost invariably within melanized capsules. As noted earlier, when melanins bond covalently with protein molecules they cross-link them and in so doing harden and strengthen the proteinaceous structures. This strengthening capability of melanin may be its role, or one of its roles, in the melanized capsules. Sooner or later encapsulated parasites die, and two causes of their death have been proposed – starvation and poisoning.

Microfilariae of *Brugia pahangi* that had been added to haemolymph from *An. quadrimaculatus* soon became encapsulated, and after 1 h some of the capsules, but not all, had become melanized. Three groups of microfilariae were transferred to vessels containing saline plus one of eight radiolabelled metabolites: (i) microfilariae in melanized capsules, (ii) microfilariae in unmelanized capsules, and (iii) microfilariae removed from infected cat blood and not encapsulated (controls). After a period of exposure, all of the radiolabelled metabolites were recovered in comparable amounts from the control microfilariae and those in unmelanized capsules. In contrast, none of the metabolites were recovered from the microfilariae in melanized capsules. Microfilariae in melanized capsules died between 12 and 24 h after the start of melanization, leading Chen and Chen (1995) to conclude that microfilariae in melanized capsules die from starvation.

It has been postulated that free radicals produced during melanin synthesis, notably during encapsulation, are toxic to invasive organisms and are a key part of the immune response. The evidence is considered in the following section.

42.10.5 Reactive nitrogen and oxygen species

These substances are free radicals, i.e. clusters of atoms one of which contains at least one unpaired electron in its outermost shell of electrons. This is an extremely unstable configuration, and free radicals quickly react with other molecules or radicals to achieve a stable configuration of electrons. They are capable of free existence under special conditions, but usually for only short periods.

(a) Reactive nitrogen species

Nitric oxide (NO) is an unstable by-product of the action of nitric-oxide synthase (NOS) on L-arginine. It is highly toxic, reacting with other compounds to generate a number of reactive nitrogen intermediates, e.g. nitrogen dioxide and peroxynitrite.

The production of reactive nitrogen species in the midgut cells of anopheline females infected with malaria parasites is described in outline here (and in detail in Volume 4, Section 51.1). Observations were made on the midgut cells of *Anopheles stephensi* or *An. gambiae* during and after their invasion by ookinetes of *P. berghei* or *P. falciparum*. Production of NOS in *An. stephensi* is regulated by a single-copy gene (Luckhart and Li, 2001). Induction of NOS occurred in midgut cells that had been invaded by ookinetes of *P. berghei*, and was followed by induction of peroxidase. While the ookinetes traversed columnar cells of *An. gambiae* by ookinetes, five peroxidase genes were expressed. It had been thought that this was

the immune response to ookinetes, but ookinetes of *P. falciparum* migrated through the columnar cells unharmed. Although NOS expression increased while ookinetes were within the cells, they had always emerged from the cells before nitration of proteins could be detected. The cells through which ookinetes had passed showed signs of damage and were expelled into the gut lumen.

It was postulated that invasion of a midgut columnar cell by an ookinete triggers a two-step process, first of NOS induction and then of peroxidase induction, and that nitrite (presumably derived from nitric oxide) and hydrogen peroxide (assumed to be present) react in the presence of peroxidase to yield nitrogen dioxide. When cell death was experimentally induced in the midgut cells, four peroxidase genes were transcriptionally activated, which suggested that peroxidase induction is part of a mechanism of programmed cell death in midgut cells. If that is the case, the produce of reactive nitrogen species in midgut cells invaded by ookinetes causes a localized apoptosis and leads to repair of the epithelium (Kumar *et al.*, 2004; Kumar and Barillas-Mury, 2005).

Ookinetes become susceptible to immune interventions when they emerge from columnar cells into the extracellular spaces of the epithelium; at this stage they may still be enclosed by the basal labyrinth or attached to the basal lamina. Within these extracellular spaces they are stationary and exposed to immune proteins from the haemolymph (Section 42.9.2).

(b) Reactive oxygen species

Reactive oxygen species are ions or very small molecules, including superoxide anion and peroxides that are highly reactive owing to the presence of unpaired valence shell electrons. Superoxide anion, O_2^-, is formed by addition of an extra unpaired electron to molecular oxygen. It is, therefore, a free radical, and is highly reactive and toxic. When two of the free radicals interact, one may be oxidized and the other reduced, forming O_2 and H_2O_2 [$2O_2^- + 2H^+ = O_2 + H_2O_2$]. That reaction is catalysed by superoxide dismutase (SOD) (EC 1.15.1.1) (Walker, 1995; Smith *et al.*, 1997). Cells usually are able to defend themselves against the actions of reactive oxygen species by enzymes such as superoxide dismutase, catalase and peroxidase.

Two reactive oxygen species, hydrogen peroxide and superoxide anion, were present in haemolymph perfused from sugar-fed, female *An. gambiae*. The concentration of hydrogen peroxide was significantly higher in the haemolymph from a resistant (R) strain than in haemolymph from females of two susceptible (S) strains. Twenty-four hours after a non-infective blood meal, the H_2O_2 concentration had increased significantly in haemolymph from the R strain and from one of the two S strains. Superoxide anion was present in the haemolymph of sugar-fed females of all three strains, but only in one of the S strains did it increase significantly after blood feeding. In other experiments, the induction in midgut tissues of mRNA of the enzymes superoxide dismutase and catalase was measured by RT (reverse transcription)-PCR. At 12 h post-blood feeding, induction of SOD mRNA had increased 1.94-fold in one of the S strains and 3.65-fold in the R strain. At 24 h post-blood feeding, induction of mRNA had fallen markedly in the R strain but continued to increase in the S strain. Induction of catalase mRNA had increased at both 12 and 24 h post-blood feeding. Kumar *et al.* (2003) concluded that the R strain of *An. gambiae* is under a chronic state of oxidative stress, which is exacerbated by the physiological stress induced by blood feeding.

In attempts to find a role for the melanin present in the capsules that form around parasites that have invaded mosquitoes, a number of investigators have proposed that toxic reactive oxygen species are produced during melanin biosynthesis. To find out whether they are produced during the early stages of melanin synthesis, the appropriate enzymatic reactions were carried out *in vitro* in the presence of electrochemical detection systems, and with the addition or not of catalase (which converts hydrogen peroxide to oxygen and water). Catalase had no effect on the rate of conversion of tyrosine

to DOPA, but it reduced the rate of oxidation of DOPA. This implicated H_2O_2 in the action of monophenol monooxygenase (EC 1.14.18.1), so-called tyrosinase; the production of H_2O_2 in the absence of catalase was confirmed analytically. Similar electrochemical response profiles showed catalase inhibition of H_2O_2 generation during oxidation of dopamine catalysed by the same enzyme. Use of the radical scavenger resveratrol revealed the involvement in both reactions of another reactive oxygen species, shown to be superoxide anion. The detection of reactive oxygen species indicated sequential univalent reductions of O_2, probably at the enzyme's copper-containing active site. Mastore *et al.* (2005) concluded that these findings point to the possible involvement of peroxidase–H_2O_2 systems and the mediation of reactive oxygen species in promoting 'melano-cytotoxic processes'.

42.11 MODULATION OF HOST IMMUNE RESPONSES BY SALIVARY PROTEINS

42.11.1 Introduction

During the initial exploratory phase of blood feeding by a mosquito, the mouthpart stylets penetrate the skin of the host, and while the mouthparts are probing, saliva is pumped into the skin through the salivary duct that runs through the hypopharynx. (Volume 1, Sections 11.2.1, 11.3.3). The saliva discharged by female mosquitoes contains many peptides and proteins, the so-called sialome.

Sequencing a full-length cDNA library from adult female *St. aegypti* salivary glands allowed identification of ten of the 11 salivary-gland proteins isolated from the same tissue by SDS-PAGE, and full-length sequence information was obtained for 32 out of 227 novel partial sequences. Two proteins of the D7 superfamily were among the most abundant of the salivary gland proteins, but their function remains unknown (Valenzuela *et al.*, 2002a,b). By analysis of the transcriptome (the complete set of RNAs) of salivary-gland cells of *An. gambiae* of both sexes, a total of 72 transcripts mainly encoding putative secretory products was identified. Most of the salivary proteins represented novel protein families and were of unknown function (Arcà *et al.*, 2005). High-throughput transcriptome analysis of adult female *St. aegypti* salivary glands yielded 614 transcripts, of which 136 were possibly salivary-gland specific. Most of the 55 proteins coded by those transcripts had no known function, but 31 genes were revealed whose expression was specific to or enriched in female salivary glands (Ribeiro *et al.*, 2007).

42.11.2 Inhibition of haemostasis

The typical tissue response of mammals to damaged blood vessels and leakage of blood, termed haemostasis, restricts the flow of blood to and out of the site of injury. Haemostasis is a threefold response involving vasoconstriction, platelet aggregation (or primary haemostasis) and a coagulation cascade (or secondary haemostasis) that results in formation of a blood clot. Saliva from mosquitoes of different genera contains a wide variety of peptides and proteins that act to counteract haemostasis in the skin of bitten animals. The saliva of all haematophagous arthropods analysed contains at least one anti-clotting, one anti-platelet and one vasodilatory substance (Schneider and Higgs, 2008).

(a) Primary haemostasis

Damage to blood vessels tears the endothelial lining, exposing sub-endothelial proteins including collagen, which almost instantly triggers platelet activation. Described very basically, in primary haemostasis circulating platelets bind to collagen, forming a plug at the site of injury. These adhesions stimulate the platelets to release the contents of their granules – which include ADP, serotonin and platelet-activating factor – so activating adherence by more platelets. Vaso-constriction is another response of damaged blood vessels (Volume 1, Section 12.2.1).

Vasodilatory constituents of mosquito saliva are thought to counter the vasoconstriction response of

the host to damaged small blood vessels. The saliva of female *St. aegypti* contains two vasodilatory peptides, sialokinin I and II, at a measured 889 pg, or 0.78 pmol, per pair of glands. When assayed for vasodilatory effects on guinea pig ileum preparations, both sialokins proved as active as the mammalian tachykinins that cause smooth muscle contraction and endothelium-dependent dilatation (Champagne and Ribeiro, 1994). Postulated enzymatic vasodilatory agents in *Anopheles albimanus* saliva were a catechol oxidase that oxidized noradrenaline and a haem peroxidase that destroyed biogenic amines released by the vertebrate host during tissue destruction. Salivary gland extracts relaxed aortic rings preconstricted with noradrenaline by the destruction of noradrenaline. Peroxidase activity present in the posterior lobes of the salivary glands was reduced after probing and blood feeding (Ribeiro and Nussenzveig, 1993).

The enzyme apyrase is among the constituents of mosquito saliva that are believed to reduce primary haemostasis. The apyrase family consists of several eukaryotic apyrases (EC 3.6.1.5) or adenosine diphosphatases. The salivary apyrases of blood-feeding arthropods are nucleoside-hydrolysing enzymes implicated in the inhibition of host platelet aggregation by the hydrolysis of extracellular adenosine diphosphate (ADP). Salivary gland homogenates from *St. aegypti* and three species of *Anopheles* hydrolysed ATP and ADP to AMP, a distinguishing characteristic of apyrase. The salivary apyrase was activated by Ca^{2+}, and had a pH optimum of 9.0. Little apyrase was detectable at emergence, but the titre reached a plateau from day 2 until at least day 10 post-emergence. It was postulated that, when carried into the host skin in saliva, the apyrase hydrolyses ADP at sites of damage to small blood vessels, so blocking ADP-dependent platelet aggregation at those sites and reducing haemostasis. At pH 7.5, apyrase activity was sufficient to account for the inhibitory effects of saliva on platelet aggregation. The three species of *Anopheles* studied showed an inverse relationship between the amount of apyrase stored in the salivary glands and the duration of probing on hosts (Ribeiro *et al.*, 1985a,b). Sequence analysis showed the apyrase from *St. aegypti* to be a slightly modified member of the family of 5'-nucleosidases (Champagne *et al.*, 1995).

The saliva of *Cx. quinquefasciatus* contains a phospholipase C for which platelet-activating factor (PAF), a phospholipid, is a specific substrate. Under *in vitro* conditions, human platelets aggregated when exposed to PAF, but aggregation was inhibited if the PAF had first been incubated for 1 min with homogenate of salivary glands from *Cx. quinquefasciatus*. In contrast, salivary gland homogenates from *St. aegypti* and *An. gambiae* did not show phospholipase C activity against PAF (Ribeiro and Francischetti, 2001).

A 6.34 kDa peptide named anophelin, isolated from salivary glands of *An. albimanus*, inhibited alpha-thrombin induced platelet aggregation. Thrombin, or fibrinogenase, is the natural coagulant of fibrinogen. Synthetic anophelin was shown to be a reversible, slow, tight-binding, specific inhibitor of thrombin (Francischetti *et al.*, 1999; Valenzuela *et al.*, 1999). Whether salivary anophelin acts rapidly enough to affect primary haemostasis has not been examined.

Saliva from female *An. stephensi* contains, in abundance, a protein named 'anopheline antiplatelet protein' (AAPP) which is secreted by the distal lateral lobes of the salivary glands. It is a specific antagonist of receptors that mediate the adhesion of platelets to collagen. Normally, collagen induces platelet aggregation by interacting with molecules of integrin $\alpha_2\beta_1$ and glycoprotein VI (GPVI) situated on the surface of platelets. The binding of AAPP to collagen effectively blocks platelet adhesion to the collagen via those two proteins, and the subsequent platelet aggregation. Integrin $\alpha_2\beta_1$ is known to mediate platelet adhesion to collagen in an Mg^{2+}-dependent manner. AAPP binds directly to collagens I and III, interfering with their interaction with glycoprotein VI on platelets, and thereby inhibits an increase in intracellular Ca^{2+}, which is an important second messenger in the platelet activation cascade. Intravenously administered AAPP inhibited collagen-induced platelet aggregation in rats (Yoshida *et al.*, 2008).

(b) Secondary haemostasis

The coagulation cascade of secondary haemostasis involves two initial pathways, the 'contact activation pathway' and 'tissue factor pathway'. These pathways are series of reactions in which proteins, several being serine proteases, become activated and catalyse the next reaction in the cascade. These 'coagulation factors' are identified by Roman numerals, with addition of a lower case 'a' to indicate an activated form, e.g. factor VIIIa (also written as FVIIIa). The two initial pathways culminate in formation of the coagulation factor Xa (FXa) and initiation of the 'final common pathway', during which thrombin is released and fibrin becomes cross-linked to form a fibrin clot around the plug of aggregated platelets.

That salivary-gland extracts from female *St. aegypti* contain an anticoagulant that acts specifically on factor Xa was shown by its inhibition of FXa-directed cleavage of a synthetic substrate. The active salivary agent was identified as a novel, proteinaceous inhibitor of serine protease activity which showed non-covalent, non-competitive inhibition kinetics (Stark and James, 1995). Later, the inhibitor was designated factor Xa (FXa)-directed anticoagulant of the yellow fever mosquito, and the gene encoding it was named *Anticoagulant-factor Xa* (*AFXa*). The protein had a predicted molecular mass of 47.8 kDa, and its amino-acid sequences showed similarities to the serpin superfamily of serine protease inhibitors (Stark and James, 1998). Coagulation tests on salivary-gland extracts from a number of mosquito species showed inhibitory actions on the 'final common pathway'. The anticoagulants from four culicine species (*St. aegypti, Stegomyia albopicta*, *Cx. quinquefasciatus*, and *Ar. subalbatus*) were FXa directed; those from four species of *Anopheles* were thrombin directed (Stark and James, 1996).

42.11.3 Examples of modulation of host immune responses

There have been many demonstrations of ingredients of mosquito saliva modulating host immune responses, both in experiments with cultured host cells and with animal hosts – mostly mice. A few of the findings are described here.

The inclusion of *St. aegypti* salivary-gland extract in cultures of naïve murine cells significantly suppressed experimentally induced production of the cytokines interleukin-2 (IL-2) and interferon gamma (IFN-γ), while the titres of other cytokines (IL-4 and IL-5) were unaffected. To determine whether salivary-gland extract could inhibit the responsiveness of cells to exogenous cytokine stimuli, the lymphocyte growth factor cytokines IL-2 and IL-4 were added to spleen cells previously treated with salivary-gland extract, and the extent of cell proliferation was measured. Cell proliferation in response to IL-2 was markedly suppressed by prior exposure of the cells to salivary-gland extract; the proliferative response to IL-4 was affected to a lesser extent. These results led Cross *et al.* (1994) to conclude that components of *St. aegypti* saliva can modulate the actions of cytokines associated with type-1 cytokine receptors, which bind IFN-γ, and enhance cellular immune responses, but not type-2 cytokine receptors, which bind IL-4 and IL-10, and enhance antibody responses. By similar arguments they concluded that components of *St. aegypti* saliva modulate the actions of cytokines associated with type-1 lymphocyte responses, which promote cytotoxic responses against intracellular pathogens, but not type-2 lymphocyte responses directed against extracellular pathogens.

In later, whole-animal experiments, mice were exposed to blood feeding by *St. aegypti* or *Cx. pipiens*, and cytokine production by stimulated lymphocytes harvested 4 to 10 days post-feeding was measured. Production of the helper T1 (T_H1) cytokine IFN-γ was significantly downregulated at 7–10 days after feeding by *Cx. pipiens* and at 7 days after feeding by *St. aegypti*. The T_H2 cytokines IL-4 and IL10 were significantly upregulated 4 to 7 days after exposure to both species. That the effects of biting by mosquitoes can be sustained for up to 10 days suggests that exposure to the natural feeding of mosquitoes can have a profound, enduring and systemic effect on T-cell functions. The immunosuppressive effect of biting by *Cx. pipiens* was not

evident when a strain of congenic, flavivirus-resistant mice was used; IFN-γ and IL-2 were significantly upregulated at days 7 and 10, correlating with a significant decrease in IL-4 at day 10. Injection of synthetic sialokinin-I into mice mimicked the effect of *St. aegypti* feeding by downregulating production of the T_H1 cytokines IFN-γ and IL-4 some 4 days after injection, but significantly upregulating that of the T_H2 cytokines IL-4 and IL-10. Injection of sialokinin-II resulted in only moderate effects on IFN-γ and IL-4 production 7 to 10 days after injection. Thus, by modulating the host T-cell response, sialokin-1 and -2 mimicked the effect of mosquito feeding (Zeidner *et al.*, 1999).

42.11.4 Immunomodulation in the context of arboviral infections

The amount of virus inoculated into the dermis of a host by an infective mosquito while probing and feeding is not insubstantial. From analysis of tissue samples removed immediately after probing and feeding on a mouse or chick by single females of four species, and taking into account unretrieved virus, the mean amounts of WNV inoculated were estimated to be: *Culex tarsalis*, $10^{4.3}$ PFU; *Cx. pipiens*, $10^{5.9}$ PFU; *Stegomyia japonica* $10^{4.7}$ PFU; and *Ochlerotatus triseriatus*, $10^{3.6}$. Where larger animals are fed on by many infective females, the amounts of virus inoculated might offset the effect of host size. When blood samples were taken from the hearts of mice or a wing vein of chicks within 5 min of the completion of blood feeding, small amounts of WNV were present in 24 of 49 samples (49%), and an early viraemia was detected in 76% of those animals (Styer *et al.*, 2007).

To find whether the inoculation of saliva by probing and feeding mosquitoes might exacerbate the pathological effects of viral infection, mice were first exposed to uninfected *St. aegypti* for 1 h, feeding on the ears either once weekly for 4 weeks or once at each of two 2-week intervals. Two weeks after the final exposure to uninfected mosquitoes, the mice were each exposed to a single mosquito that had been parenterally infected with WNV. The prior exposure of mice to mosquito feeding increased mortality following WNV infection, and more intensive pre-exposure resulted in higher death rates. Batches of mice that were exposed to ~17 uninfected mosquitoes either two or four times over the course of a month suffered 68% or 91% mortality, respectively, compared with 27% mortality in unexposed mice ($p < 0.01$) (Schneider *et al.*, 2007).

To test whether cytokine expression differed between groups of mice, total RNA was isolated from the biting sites and draining lymph nodes and the relative titres of IL-2, IL-4, IL-10, IL-12p40, IFN-β, and IFN-γ were measured. In three experiments, at 36 h post-infection, IL-10 expression was elevated in the dermis and draining lymph nodes of sensitized mice as compared with naïve mice ($p < 0.04$). This immunoregulatory cytokine is multifunctional, and could have created favourable conditions for viral replication in a number of ways. The expression of IL-4 was 50-fold higher in the lymph nodes of sensitized mice, and, although not statistically significant, the consistency of the increased expression suggested a trend towards elevated T_H2 responses in pre-exposed mice. Passive transfer of serum from exposed mice to naïve mice resulted in elevated mortality, thus implicating the humoral immune response in the modification of pathogenesis in mosquito-sensitized mice. That was thought not to preclude the possibility that the cellular response is also important.

Histological examination of skin from the biting sites and of tissue from draining lymph nodes, at 24 and 48 h post-infection, revealed only a transient, mild inflammatory reaction in naïve mice, in contrast to significant cellular infiltration and a >2-fold increase in tissue size in pre-exposed mice. The oedema at the biting site in sensitized mice was characterized by mononuclear cell and neutrophil infiltrates. These results supported the hypothesis that an immune response of the host to salivary proteins promotes recruitment of cells to the inoculation site (Schneider *et al.*, 2007). A response of that type would help protect the host against invasive cellular organisms, but not against

arboviruses when the host cells are susceptible to infection. These results indicated that constituents of the saliva of adult female mosquitoes not only function to reduce the haemostatic responses of their hosts during blood feeding, but also, after the completion of blood feeding, affect immune responses of the host that normally are directed against invasive pathogenic agents.

In *in vitro* experiments, *St. aegypti* saliva decreased the expression of IFN-β and inducible nitric oxide synthase in macrophages, by up to 50% and 70%, respectively, while transiently enhancing IL-10 expression. *In vivo* experiments indicated that the predominant effect of *St. aegypti* feeding on mice was to significantly reduce the recruitment of T-cell lymphocytes, such that mice that had previously been exposed to the mosquito bites had up to 2.8-fold fewer T-cells upon inoculation with WNV than mice that had not been previously exposed to bites when inoculated. These shifts in the T-cell population were associated with significantly elevated IL-10 (up to fourfold) and WNV (up to tenfold) in the skin and draining lymph nodes, and suggest that mosquito saliva had deregulated antiviral signalling by 'antigen-presenting cells'. They revealed a possible mechanism for the observed enhancement of WNV-induced disease mediated by mosquito saliva via a reduction of T-cell lymphocyte and antiviral activity at the inoculation site, an elevated abundance of susceptible cell types, and a concomitant increase in the immunoregulatory activity of IL-10 (Schneider *et al.*, 2010).

43

Viruses

43.1 INTRODUCTION

43.1.1 Composition, structure and replication of viruses

The infectious particle of viruses (or virion) consists of a core of nucleic acid (the genome) and a protein shell (the capsid). The nucleic acid core and capsid together constitute a nucleocapsid. In virions with a spherical form, the capsid has the form of an icosahedron (see Glossary, Appendix 3). In helical virions, the nucleic acid is interwoven among repeated capsid proteins. With some virus families, the nucleocapsids are enveloped, i.e. surrounded by a bilayered, lipoprotein 'envelope'. Usually, proteins that extend outwards from the envelope are glycosylated. The envelope enhances the stability and persistence in the environment of viruses, and functions in cell penetration. In viruses of the family *Baculoviridae*, subfamily Entomopoxvirinae (Poxviridae) and genus *Cypovirus* (Reoviridae), the virions are enclosed within large virus-encoded, proteinaceous occlusion bodies. Depending on the group, a single occlusion body may contain from one to several thousand virions (Harper, 1998; Fauquet *et al.*, 2005).

The first step in penetration of a cell is the binding of 'attachment protein' molecules on the surface of the virus to specific 'receptor' molecules on the cell's surface. Once bound, some viruses are internalized by the cell itself, either by translocation across the plasma membrane or by the plasma membrane invaginating to form a vacuole (the process of receptor-mediated endocytosis; Volume 1, Section 20.4.1). In some other cases, a reaction between an attachment protein and the plasma membrane allows the virus to fuse with the plasma membrane and release its nucleocapsid directly into the cytoplasm.

The first stage in virus multiplication is replication, i.e. duplication of the genome. The method of replication varies according to the size of the genome and its chemical nature. Viruses with a large genome can produce many proteins, certain of which assist the process of replication; they also permit the formation of a complex virion. Viruses with a small genome have limited capabilities; for replication they depend on cellular enzymes, and their virion structure is much simpler. Viral genomes are composed of either DNA or RNA, and in both cases of either one strand or two strands. Nucleic acid strands that are protein coding, i.e. that have the same base sequence as the specific mRNA, are described as positive sense; strands that have a complementary configuration are described as negative sense. In a few virus families, a single-stranded genome contains information encoded in both the positive-sense and the negative-sense configurations, and is described as ambisense. The unit of measurement used to describe the size of single-stranded

genomes is the kilobase (kb), i.e. 1000 nucleotide residues; that used for double-stranded genomes is kilobase pair (kbp).

Replication of both RNA and DNA genomes occurs through the formation of complementary strands. For most RNA viruses this process is catalysed by specific RNA replicases. In the case of negative-strand RNA viruses the replicases are always, and necessarily, packaged into the capsid because the infecting strand does not code for protein. In contrast, the RNA of positive-strand RNA viruses can serve as mRNA, so the naked genome itself is infectious. Depending on their genome size and coding capability, DNA viruses replicate in either the host-cell nucleus or the cytoplasm.

Viruses with an **ssRNA genome** have a very small genome. Single-stranded (ss)RNA genomes may be (+) sense or (–) sense or, rarely, ambisense. A (+)sense ssRNA genome is infectious in itself, since by functioning as an mRNA it can be translated to produce RNA transcriptase and other enzymes after its entry into the cell. Viruses with (–)sense ssRNA genomes complementary to mRNA cannot function as mRNAs. However, an RNA-transcriptase is carried in the nucleocapsid, and, once it is within a cell, both mRNAs and genomic copies can be made.

Viruses with a **dsRNA genome** also have a small genome. The core is transcriptionally active and transcribes the (–)sense RNA to (+)sense mRNAs, which are released from the core to provide mRNAs for both protein synthesis and as virion components that are encapsidated into virion precursors.

Viruses with an **ssDNA genome** have a very small genome too. They must enter a nucleus and be copied to produce a dsDNA replicative form, which can be used to produce both mRNA and progeny genomes. However, only actively dividing cells will support their replication, because cells that are not actively dividing do not make DNA.

Viruses with a **dsDNA genome** have a large genome with an extensive coding capability. A capability of producing DNA or RNA from a DNA template enables some of them to use polymerases that are present in the cell nucleus. Some others, such as the pox viruses, set up a so-called 'second nucleus' in the cytoplasm, where replication occurs (Harper, 1998).

The later stages of virus development involve formation of the capsid, assembly of the various parts into a virion, acquisition of an envelope, and escape from the host cell. In general, more complex viruses require more complex assembly pathways. Viral protein synthesis can take place only in the cytoplasm, and most viruses are assembled there. The membranous part of the virion envelope is acquired from one of a number of specific cellular membranes, depending on the virus. The virion escapes into the extracellular environment by 'budding' through the plasma membrane; and the envelope may be acquired at that stage.

43.1.2 Classification and nomenclature

Viruses are classified into the hierarchical grades of order, family, subfamily, genus and species, but for many viruses the family is the highest ranked taxon, and not all families are divided into subfamilies. The fundamental taxonomic grouping is based on the chemical and physical nature of the genome (Table 43.1). Classification into families involves such characters as the configuration of the genome (whether linear, coiled, supercoiled or segmented), whether or not the virion is enveloped, and whether the hosts are invertebrates, vertebrates, plants or of another taxon.

The rules of virus taxonomy are more prescriptive than those for animal taxonomy, and it could be helpful to those even peripherally involved with virus nomenclature to have a working knowledge of its basis. Rules on the nomenclature of viruses are provided in 'The International Code of Virus Classification and Nomenclature of ICTV', most recently published in *Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses* (ICTV) (Fauquet *et al.*, 2005). They include the following seven key statements. (i) A virus species is defined as a

Table 43.1 Classification of viruses based on the chemical and physical characteristics of the genome. Certain viruses of the named families infect mosquitoes. Most mosquito-borne arboviruses are ssRNA viruses, but the dsRNA family *Reoviridae* includes accepted or probable arboviruses in the genera *Orbivirus* and *Seadornavirus* (Table 44.1).

DNA viruses

Double-stranded (ds)

Comprises one positive-sense (+) strand (containing the same base sequence as the mRNA) and one negative-sense (–) strand (containing base sequences complementary to those of the mRNA). Size, 5–375 kbp. Includes the families *Baculoviridae*, *Iridoviridae* and *Poxviridae*.

Single-stranded (ss)

Composed of one positive-sense strand which contains the same base sequence as the mRNA. Size, 2–9 kb. Includes the family *Parvoviridae*.

RNA viruses

Double-stranded (ds)

Composed of one positive-sense and one negative-sense strand. The genome is segmented, and is composed of 2–12 dsRNA molecules. Size, 3–32 kbp. Includes the family *Reoviridae*.

Single-stranded (ss)

The genome consists of a single, unsegmented RNA molecule which may be positive sense or negative sense. Size, 2–31 kb.

(i) Positive-stranded; one positive-sense strand which can function as mRNA. Includes the families *Flaviviridae*, *Nodaviridae* and *Togaviridae*.

(ii) Negative-stranded; one negative-sense strand which is complementary to the mRNA produced from it. Includes the families *Bunyaviridae* and *Rhabdoviridae*.

polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche. (ii) The name of a taxon has no official status until it has been approved by the ICTV. van Regenmortel (2008) clarified that statement by defining two terms used in it. 'A polythetic class consists of members that exhibit overall similarity and have a large number of common properties. However, the members of a polythetic class do not share a common character that constitutes a single defining property of the class'. 'The ecological niche of a virus refers to certain biological properties of the virus, such as host range, tissue tropism in the host, and type of vector, rather than a geographical space or particular environment'.

Proposals for new names or name changes must be submitted to the Executive Committee of the ICTV, when they will be examined by specialist groups. (iii) Proposed names are 'valid' if they conform to the Rules set out in the Code, and valid names become 'accepted names' if they are recorded as approved in an ICTV Report. (iv) In formal taxonomic usage, the accepted names of virus Orders, Families, Subfamilies, Genera and Species are printed in italics and the first letters of the names are capitalized. (v) The term for the taxon grade should precede the name of the taxonomic unit; thus ... the family *Bunyaviridae* ... the genus *Orthobunyavirus* ... the species *California encephalitis virus*. (vi) For species names, words after the first do not have the first letter capitalized unless they are proper nouns: thus, *Yellow fever virus* but *Murray River encephalitis virus*. (vii) Family names end with the suffix *viridae*, and subfamily names with *virinae*; both genus names and species names end with the suffix *virus*.

Comparison of the approved virus names listed in the last three Reports of the ICTV, the Sixth (Murphy *et al.*, 1995), Seventh (van Regenmortel *et al.*, 2000) and Eighth (Fauquet *et al.*, 2005), reveals a rationalization of viral systematics followed by continuing but moderate change. In this volume, the classification of viruses and the names used for viruses are those provided in the Eighth Report and available online in 'The Universal Virus Database of the International Committee on Taxonomy of Viruses' (ICTVdb) (http://www.

ICTVdb.org/), with the addition of viruses subsequently officially recognized.

It is a philosophical perception of virus taxonomy that 'Real objects located in space and time such as viruses must be distinguished from abstractions like virus species that exist only in the mind'. Also, that 'tangible objects like viruses, i.e. concrete individuals' should be distinguished from 'the abstract classes of virus species used by taxonomists, which are mental constructs used for building up a classification' (van Regenmortel, 2006, 2008).*

In virus taxonomy the distinction between the abstract and the tangible is brought about by the use of a 'formal nomenclature' for the species names and higher taxon names that *represent* taxonomic units, in which case the names are italicized and the initial letter is capitalized. Clause 3.40 of 'The International Code of Virus Classification and Nomenclature' states 'Taxa are abstractions and thus when their names are used formally, these are written distinctively using italicization and capitalization. In other senses, such as an adjectival form ... italics and capital letters are not needed'. After any name has been written once in the style of formal nomenclature, subsequent usages may be informal. Informal names, irrespective of rank, are not italicized and are written entirely in lower case; only proper names are given an initial capital letter. The informal name of the taxon should not end with the formal suffix, and the grade of the taxon should follow the taxonomic name; thus ... the baculovirus family ... the phlebovirus genus ... the Rift Valley fever virus. The names of tentative or unassigned species, or of serotypes or strains, should not be italicized. Thus, *Aroa virus* includes the serologically distinguishable Bussuquara virus, Iguape virus and Naranjal virus. Where a virus has been known by more than one name, the eventually unaccepted names become synonyms; for example Tipula iridescent virus is a synonym of *Invertebrate iridescent virus 1* (IIV-1) (Fauquet *et al.*, 2005).

For many years, virus species were distinguished by their serological characteristics, and major infraspecific taxa were classed as 'serotypes'. *California encephalitis virus* (genus *Orthobunyavirus*) comprised 13 well-characterized serotypes, which differed in geographical range, host species, toxicity to humans (as dead-end hosts) and in other ways. Local populations of a species or of a serotype that were distinguishable were classed as 'strains'. With the advent of molecular biology, information on the molecular structure of serotypes became available, and this very largely supported their identities as distinct subspecific taxa. Comparison of the Seventh Report (2000) and Eighth Report (2005) of the ICTV shows that all of the former 13 serotypes of *California encephalitis virus* are treated as strains. The term 'strain' is now used both for what were classed as serotypes and for distinguishable local populations.

Every accepted virus species and accepted serotype/strain is assigned an abbreviation, which often is an acronym: that for *California encephalitis virus* (as a strain of that species) is CEV, while that for its serotype/strain La Crosse virus is LACV.

Newly described viruses have no official status and are not recognized as virus species until they have been submitted to the Executive Committee of the ICTV in the form of a taxonomic proposal and approved (Clause 3.20 of the International Code). The names of 'Accepted species' are kept by the ICTV in an Index and published in successive Reports. If after submission to the ICTV the assignment of a new virus to a genus remains unclear, it is termed a 'Tentative species' of that genus. New viruses that resemble recognized members of a family but that are sufficiently different to suggest that they may represent new genera are termed 'Unassigned viruses'.

Because the highly variable nomenclature of viruses 'defies direct and systematic interrogation in a data base', a Decimal Code of virus nomenclature was first used on a substantial scale in *The Springer Index of Viruses* (eds C.A. Tidona and

* Distinction between the actual and the abstract was explored by the surrealist artist René Magritte, first in a painting completed in 1929 and entitled *La trahison des images* (The treachery of images). The painting showed a smoker's pipe and immediately below it the words *Ceci n'est pas une pipe*. Later, Magritte clarified this distinction with the comment 'L'image d'une pipe n'est pas une pipe'.

G. Darai, 2002), and later was adopted for the ICTV database. The code comprises up to 19 digits, indicating the systematic position of viruses from the rank of order down to that of isolate. For many viruses, the digits reserved for the ranks order, subfamily or subspecies are redundant. The following decimal code nomenclature of *Invertebrate iridescent virus 3* is taken from the ICTV database.

Taxon level	Decimal code
Order	00 = (not assigned)
Family	00.036 = *Iridoviridae*
Subfamily	00.036.0 = (not assigned)
Genus	00.036.0.02 = *Chloriridovirus*
Type species	00.036.0.02.001 = *Invertebrate iridescent virus 3*

(See Section 45.2.1 for the more extensive decimal code for the species and strains (serotypes) of the Dengue virus group.)

43.2 CHARACTERISTICS, SYSTEMATICS AND BIOLOGY BY FAMILY OF VIRUSES THAT INFECT CULICIDS AND ARE NOT ARBOVIRUSES

Seven virus families are known that include groups of species that infect culicids but are not arboviruses, i.e. their mosquito hosts are not biological vectors that transmit the viruses to other organisms (Table 43.2). In three of the families, *Baculoviridae*, *Reoviridae* (genus *Cypovirus*) and *Poxviridae* (subfamily *Entomopoxvirinae*), the virions are enclosed within occlusion bodies. In the other four families, *Flaviviridae*, *Iridoviridae*, *Nodaviridae* and *Parvoviridae*, the virions are not occluded. In this section, the example of Becnel (2007) is followed in changing the names of some viruses, and the abbreviations of those names, in accordance with changes in mosquito nomenclature. As one example, *Aedes sollicitans* NPV (AesoNPV) has become *Ochlerotatus sollicitans* NPV (OcsoNPV).

Certain mosquito species, notably *Culex pipiens*, *Culex quinquefasciatus* and *Stegomyia albopicta*, are infected with the symbiotic bacterium *Wolbachia*, which in turn is infected with bacteriophages – tailed viruses of the Order *Caudovirales* (Section 46.9.8).

43.2.1 Family *Baculoviridae*

Baculoviruses are a group of arthropod-specific viruses with rod-shaped nucleocapsids of 30–60 nm × 250–300 nm. There are two virion phenotypes. (i) Occlusion-derived virions (ODVs) are occluded in a crystalline protein matrix, the occlusion body (OB), and initiate infection in the midgut epithelium of infected insects. Each OB encloses single or multiple enveloped nucleocapsids. (ii) Budded virions (BVs) are produced after initial infection of a host and bud through the plasma membrane of infected cells. They typically contain a single nucleocapsid within an envelope that is derived from the host plasma membrane and modified by one or more viral proteins. These two forms reflect their respective roles in cell-to-cell (BV) and insect-to-insect (ODV) transmission of baculovirus infection.

All baculoviruses produce occlusion bodies that contain infectious, lipid enveloped, rod-shaped virus particles, but they occur in two structural forms which correlated with an earlier classification of baculoviruses into two genera. Species of the genus *Nucleopolyhedrovirus* (nucleopolyhedroviruses, NPVs) had polyhedral occlusion bodies of 0.15 to 30 μm, which characteristically contained many ODVs. Species of the genus *Granulovirus* (granuloviruses, GVs) had smaller, often ovoid occlusion bodies of 0.13 × 0.50 μm, which normally contained a single ODV. Both genera had predominantly lepidopteran hosts. Phylogenetic analyses revealed the evolution of four clades with specific host types, and led to a proposal that the species be grouped into four genera: *Alphabaculovirus*, NPVs with lepidopteran

Table 43.2 Viruses that infect culicids and that are not arboviruses, with their assigned abbreviations. The names of genera and species that have been *formally approved by the ICTV are italicized; the names of viruses that are tentative species of a genus are not italicized. The list also includes five pox viruses (Poxviridae) that have been shown experimentally to be mechanically transmitted by adult mosquitoes but that do not infect those mosquitoes.

Family	*Genus or other taxon*	*Viruses isolated from culicids*	*Assigned abbreviation*
Baculoviridae	*Deltabaculovirus*	*Culex nigripalpus NPV* †	CuniNPV
		Ochlerotatus sollicitans NPV §	OcsoNPV
		Uranotaenia sapphirina NPV	UrsaNPV
Flaviviridae	*Flavivirus*	Cell fusing agent virus	CFAV
		Kamiti River virus	KRV
		Culex flavivirus	CxFV
Iridoviridae	*Chloriridovirus*	*Invertebrate iridescent virus 3* †	IIV-3
Nodaviridae	*Alphanodavirus*	*Nodamura virus* †	NoV
Parvoviridae	*Brevidensovirus*	*Stegomyia aegypti densovirus* †§	SaeDNV
		Stegomyia albopicta densovirus §	SalDNV
		Anopheles gambiae densovirus	AgDNV
		Culex pipiens pallens densovirus	CppDNV
		Haemagogus equinus densovirus	HeDNV
	Cupidensovirus	*Culex pipiens densovirus* †	CpDNV
Poxviridae	*Avipoxvirus*	*Fowlpox virus* †	FWPV
		Turkeypox virus	TKPV
	Leporipoxvirus	*Myxoma virus* †	MYXV
		Rabbit fibroma virus	RFV
		Squirrel fibroma virus	SQFV
	Gammaentomopoxvirus	*Stegomyia aegypti entomopoxvirus* §	SAEV
Reoviridae	*Cypovirus*	Cypovirus-17	UsCPV-17
		Culex restuans cypovirus	CrCPV-17
	Dinovernavirus	*Stegomyia pseudoscutellaris reovirus* †§	SPRV

*, Formally approved by publication in the Universal Database of the ICTV (http://www.ictvdb.org/) or in the document '*ICTV Official Taxonomy: Updates since the 8th Report*' (accessible at http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/default.aspx). Where the virus name is not italicized, the virus has not been formally approved.
†, Type species of the genus.
§, Name of host genus changed from *Aedes* to *Stegomyia* in the revised nomenclature of the Aedini. The table also lists five pox viruses (Poxviridae) that have been shown experimentally to be mechanically transmitted by adult mosquitoes but that do not infect those mosquitoes.

hosts; *Betabaculovirus*, GVs with lepidopteran hosts; *Gammabaculovirus*, NPVs with hymenopteran hosts; and *Deltabaculovirus*, NPVs with dipteran hosts (Afonso *et al.*, 2001; Herniou *et al.*, 2004; Jehle *et al.*, 2006). In 2006 and 2007, those four genera received formal recognition ('*ICTV Official Taxonomy: Updates since the 8th Report*', http://talk.ictvonline.org/files/ictv_official_taxon omy_updates_since_the_8th_report/m/default.aspx).

(a) *Characteristics of the family* Baculoviridae

Genome. Nucleocapsids contain a single circular, supercoiled dsDNA molecule of 80–180 kbp. The genomes of many baculoviruses have been sequenced, including that of *Culex nigripalpus* (CuniNPV). *Virion*. Rod-shaped; average diameter 30–60 nm, and average length 250–300 nm; enveloped. A single virus species may express two virion phenotypes: budded and occluded. *Replication*. Replication of NPVs takes place within the host nucleus, that of GVs within a nucleocytoplasmic stroma after disintegration of the nuclear membrane. Gene expression occurs during three time periods during an infection – early, late and very late – and is regulated primarily at the level of transcription. During the earlier stages of infection, replicated genomes become packaged with viral proteins to form nucleocapsids. Budded virions are generated when nucleocapsids bud through the plasma membrane at the surface of infected midgut cells, acquiring a virally modified plasma membrane envelope. Budded virus is the infectious unit that spreads the infection to other host tissues. During later stages of infection, viral genes that encode for occlusion matrix proteins are hyper-expressed, and those proteins form a matrix, in most cases crystalline, that embeds nucleocapsids within occlusion bodies. The occlusion bodies of three genera are surrounded by an envelope the source of which is not clear. The occlusion bodies of nucleopolyhedroviruses may be polyhedral or amorphous, range in size from 0.15 to 15 μm, and contain many virions. Those of granuloviruses are ovicylindrical, about 0.3 × 0.5 μm in size, and contain only one or two virions. *Hosts*. Baculoviruses are known only from arthropods – overwhelmingly from insects, but a few are known from shrimps (Crustacea). *Infection*. Disintegration of the host cadaver leads to the release of occlusion bodies and possible horizontal transmission between hosts. The occlusion bodies are thought to protect virions from environmental degradation until they are ingested by a host. Virions are released within the midgut lumen and infect midgut epithelial cells, entering them by endocytosis (Federici, 1997; Boucias and Pendland, 1998; Friesen and Miller, 2001; Herniou *et al.*, 2003; Fauquet *et* al., 2005; Jehle *et al.*, 2006).

(b) *Characteristics of the genus* Deltabaculovirus

The dsDNA genome of *Cx. nigripalpus NPV* (CuniNPV), the type species of *Deltabaculovirus*, is small and was predicted to comprise 109 genes. Although 36 genes show homology to genes from other baculoviruses, their orientation and sequence reveal little conservation relative to those of the lepidopteran baculoviruses, indicating a large evolutionary distance between them. The absence of homologues of the occlusion-derived virion envelope proteins and occlusion body proteins of the lepidopteran baculoviruses suggests functional, structural and compositional differences (Afonso *et al.*, 2001).

Deltabaculoviruses infect midgut tissues of larval mosquitoes, and principally the epithelium, but infections have been found in adult midguts also. In infected larvae, whitish cysts or nodules, which are the hypertrophied nuclei of epithelial cells and contain occlusion bodies, occur either throughout the midgut or in just one or two regions of it. The occlusion bodies (OBs) of deltabaculoviruses are either polyhedral in shape and some 5–20 μm in size, or globular and only 0.5 μm in size. Typically, each contains four rod-shaped virions of about 60 × 250 nm, but sometimes up to eight (Becnel, 2007). The OBs of different species of *Deltabaculovirus* differ substantially from one another. Mature occlusion bodies of CuniNPV are uniform, globular particles of c. 400 nm diameter, and lack the polyhedron envelope that surrounds the OBs of most other baculoviruses. The mass of the occlusion body

protein, at ~90 kDa, is about three times that of species of the other genera, and it shares no sequence similarity with any other known protein (Perera *et al.*, 2006).

In OcsoNPV, mature virions were 80 × 220 nm in size and surrounded by a 12.7-nm thick, bilayered membrane. Occlusion started as soon as mature virions appeared, with deposition of material around single virions, and later the OBs enlarged to incorporate additional virions. Sectioning of OBs revealed a regular crystal-lattice structure. Between 18 and 30 h post-infection the host nuclei contained from 10 to 40 OBs, which were irregularly oval in shape and approximately 1–2 μm in size. By 30 h the infected nuclei were very large and opaque due to the accumulation of polyhedra (Stiles *et al.*, 1983). In UrsaNPV, some virions became occluded singly, when protein was deposited around them, whereas other virions became attached to existing pleomorphic occlusion bodies. Small occlusion bodies coalesced to form dumbbell or bow-tie shaped bodies (Figure 43.3D). Mature occlusion bodies were up to 10–15 μm in length and 3–4 μm in diameter, and contained many enveloped, rod-shaped virions. They did not have a crystalline lattice structure and lacked an envelope (Shapiro *et al.*, 2004).

When larvae of *Uranotaenia sapphirina* and *Uranotaenia lowii* in deionized water were exposed to UrsaNPV none became infected, but in 10mM $CaCl_2$ the infection rates were 12.9% and 14.5%, respectively (Shapiro *et al.*, 2004). Deltabaculoviruses are highly virulent. Death of *Cx. nigripalpus* and *Cx. quinquefasciatus* larvae usually occurred by 72–96 h post-infection, by which time most nuclei in the gastric caeca and posterior stomach were infected (Moser *et al.*, 2001). Larvae of *Georgecraigius epactius* infected with UrsaNPV were dying or dead within 40 h, and later necrosis of the midgut cells released OBs into the midgut lumen (Stiles *et al.*, 1983).

(c) *Culex nigripalpus nucleopolyhedrovirus (CuniNPV)*

CuniNPV was discovered infecting *Culex* larvae in two highly eutrophic water bodies ('pools') in Florida. One was a man-made pond in Alachua County which received swine effluent, and contained *Cx. nigripalpus* or *Cx. quinquefasciatus* larvae during different seasons of the year. The other was a so-called lagoon in Marion County which received dairy effluent, and contained *Cx. quinquefasciatus* as the predominant species, with *Cx. nigripalpus* larvae appearing infrequently. During a 2-year period, CuniNPV caused frequent epizootics in *Culex* larvae in the Alachua County pool, but only five small outbreaks in the Marion County pool. Analyses of water from the two pools showed a high salt content, notably of Mg^{2+} and Ca^{2+}. Laboratory tests on the effects of individual cations on the rate of infection of *Culex* larvae with CuniNPV demonstrated the importance of those divalent ions: over the range 1.8–12.5 mM, Mg^{2+} was an activator of infection; at concentrations of 0.5 mM or more, Ca^{2+} reduced infection rates (Table 43.3). Larvae of *Culex salinarius* and *Culex restuans* were present in the pond in Alachua County during the winter, but at low density. Larvae of *Cx. salinarius* showed high infection rates with CuniNPV, whereas larvae of *Cx. restuans* were never infected (Becnel *et al.*, 2001).

The larvae of 12 mosquito species present in north-eastern USA were tested for susceptibility to infection by CuniNVP. Larvae of four species of *Culex* proved susceptible – *Cx. pipiens, Cx. quinquefasciatus, Cx. salinarius* and *Cx. restuans* –those of *Cx. restuans* being the least susceptible. Larvae of the following species were not susceptible: *Culex* (*Neoculex*) *territans*; *Aedimorphus vexans*; *Culiseta morsitans*; and five species of *Ochlerotatus* – *Ochlerotatus canadensis, Ochlerotatus cantator, Ochlerotatus excrucians, Ochlerotatus stimulans* and *Ochlerotatus triseriatus*. For most species, the test larvae were wild caught (Andreadis *et al.*, 2003).

When third-instar *Cx. quinquefasciatus* larvae were exposed to infection, 62.5% of the larvae shown to be infected pupated, of which 50.9% died before emergence. Electron microscope examination of some adults surviving at 24 h post-emergence revealed occlusion bodies within nuclei of midgut epithelial cells and in meconium in the gut lumen. Becnel *et al.* (2003) surmised that adult male and female mosquitoes could spread CuniNPV to new

Table 43.3 Effects of the divalent ion content of two eutrophic pools in Florida on the epizootiology of *Culex nigripalpus NPV* (CuniNPV) infections of larvae of *Culex nigripalpus* and *Culex quinquefasciatus*. (From the data of Becnel *et al.*, 2001.)

A. Eutrophic pools

Location of eutrophic pool	*Contaminants*	*Cation concentrations (mM)* K^+	Na^+	Mg^{2+}	Ca^{2+}	*Type of CuniNPV infections*
Alachua County	Swine effluent	10.7 ± 0.5	3.2 ± 0.2	1.9 ± 0.1	0.8 ± 0.05	Frequent epizootics
Marion County	Dairy effluent	5.8	3.4	3.7 ± 0.3	3.0 ± 0.9	Occasional outbreaks

In the pool in Alachua County, *Cx. nigripalpus* larvae were abundant during the warmer months (May–November), whereas *Cx. quinquefasciatus* larvae were abundant during the cooler months (December–April). In the pool in Marion County, *Cx. quinquefasciatus* was the dominant species while *Cx. nigripalpus* was found infrequently.

B. Laboratory assays with *Cx. quinquefasciatus* larvae

Culture medium	*Distilled water*	*Distilled water* + 1.8 mM Mg^{2+}	*Distilled water* + 1.8 mM Mg^{2+} + 0.5 mM Ca^{2+}	*Swine effluent*	*Dairy effluent*
Infection rate (%)	0.2 ± 0.1	10.4 ± 5.2	3.7 ± 1.9	16.2 ± 2.4	0.1 ± 0.1

aquatic habitats by voiding meconium containing infectious OBs into the aquatic environment, or by the death there of infected adults. Mosquitoes void the semi-solid meconium 1–2 days after emergence (Volume 1, Section 16.4).

In *Culex nigripalpus* larvae infected with CuniNPV, viral replication and multiplication take place only in the alimentary canal, where they occur almost exclusively in epithelial and regenerative cells of the posterior midgut and the proximal region of the gastric caeca The cells of the cardia are never infected, and those of the anterior midgut are seldom infected (Figure 43.1). Becnel (2006) surmised that, following the ingestion of occlusion bodies, virions are released in the highly alkaline conditions of the midgut lumen. However, the OBs of CuniNPV were very resistant to dissolution at pH 12.0 (Moser *et al.*, 2001), while the highest alkalinity measured in the midgut lumen of *Cx. pipiens* larvae was pH 10–10.5 (Dadd, 1975).

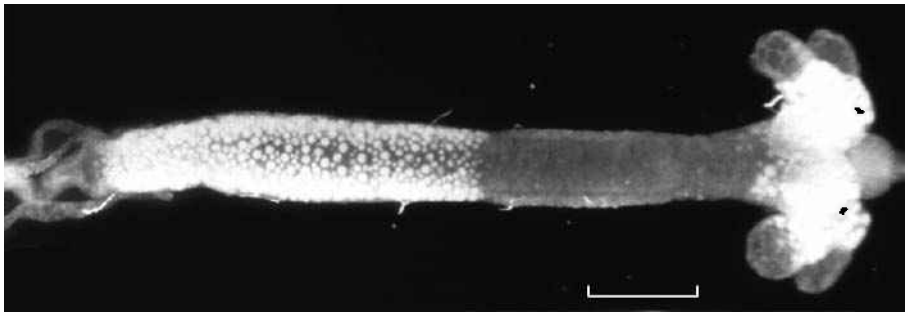

Figure 43.1 Midgut of *Culex nigripalpus* larva under phase-contrast illumination, showing evidence of infection with *Culex nigripalpus NPV* (*Baculoviridae*). Nuclei within infected cells in the proximal region of the gastric caeca and the posterior region of the stomach appear white due to the proliferation of occlusion bodies. Scale bar 0.5 mm. (Reproduced from Moser *et al.* (2001) with permission.)

The first developmental phase starts when virions enter midgut cells, presumably through the apical plasma membrane. Later, nucleocapsids are seen dispersed throughout the nucleoplasm. Naked nucleocapsids, singly or in groups, bud outwards through the nuclear envelope, regions of which form transport vesicles around them (Figure 43.2D). Once within the cytoplasm, the nucleocapsids of the budded virions are released and accumulate at the cell margins, and are thought to infect other midgut cells. In the second developmental phase, infected host-cell nuclei show a central virogenic stroma within which arrays of nucleocapsids appear (Figure 43.2A,B). The stroma develops into dense structures, distributed throughout the nucleoplasm, which are involved in the occlusion of virions, and eventually occlusion bodies fill the nucleus. There is no evidence of coalescence of smaller occlusion bodies. The nuclear envelope remains intact throughout the process.

The developmental sequence involves two virion phenotypes: an occluded form (ODV) that initiates infection in the midgut epithelial cells; and a budded form (OB) that spreads the infection in the midgut. (In other nucleopolyhedroviruses the budded virions spread the infection to other tissues.) The mature occlusion bodies are amorphous, approximately 400 nm in diameter, and typically contain four to six enveloped, rod-shaped virions (Figure 43.2C). The OBs lack the envelope characteristic of other nucleopolyhedroviruses. All other nucleopolyhedroviruses encode highly conserved OB proteins, called polyhedrin, with molecular weights that vary by ±29 kDa. At ~90 kDa, the OB protein of CuniNPV is three times larger. The CuniNPV genome does not contain a gene that might encode polyhedrin, and the amino acid sequence of its OB protein does not show sequence similarity to any protein in the available database (Afonso *et al.*, 2001; Moser *et al.*, 2001; Perera *et al.*, 2006).

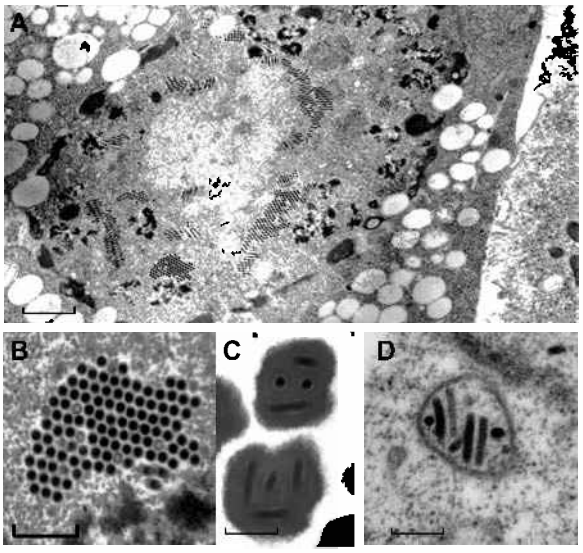

Figure 43.2 Details from electron micrographs of midgut cells of *Culex nigripalpus* larvae infected with Culex nigripalpus NPV (CuniNPV). **A**. Section through part of a nucleus during the second phase of virogenesis. A centrally located virogenic stroma is surrounded by arrays of nucleocapsids, and heterochromatin is distributed over the inner surface of the nuclear envelope. Vacuoles accumulate in the cytoplasm. Scale bar 1.0 µm. **B**. Small array of nucleocapsids from the same preparation as **A**. Scale bar 0.20 µm. **C**. Occlusion bodies formed during the second phase of virogenesis. The occlusion bodies contain rod-shaped virions, each consisting of nucleocapsid, intermediate layer and envelope. Scale bar 0.4 µm. **D**. Section through a transport vesicle containing nucleocapsids after it has budded through the nuclear envelope. Observed within an epithelial cell of the posterior stomach region during the first phase of virogenesis. Scale bar 0.25 µm. (Reproduced from Moser *et al.* (2001) with permission.)

(d) Ochlerotatus sollicitans NPV (OcsoNPV)

Reports of baculoviruses infecting wild mosquitoes are uncommon, and epizootics in natural populations are rarely observed. The first nucleopolyhedrovirus found infecting mosquitoes was in wild-caught larvae of *Ochlerotatus sollicitans* (formerly *Aedes sollicitans*), and it was then named Aedes sollicitans NPV. The infected larvae were sluggish, and the gut wall was visibly white due to the presence of occlusion bodies within the nuclei. Placing uninfected with live infected larvae led, on average, to 29% of the former becoming infected. At 22 °C, the virus had a prepatent period of 24–48 h, and infected larvae survived no more than 32 h after infections were apparent. Of 33 wild-caught adult females of *Oc. sollicitans* that laid viable eggs, one female gave rise to 56 larvae, of which one was infected with OcsoNPV, an apparent case of vertical transmission (Clark *et al.*, 1969; Clark and Fukuda, 1971).

Exposure of mosquito larvae to a macerate of infected *Oc. sollicitans* larvae revealed susceptibility to infection with OcsoNPV in larvae of *Oc. triseriatus* and *Ochlerotatus tormentor*, but not in *Ochlerotatus taeniorhynchus* or *Jahnellius sierrensis* (formerly *Ochlerotatus sierrensis*). Larvae of *Psorophora varipes* and *Psorophora ferox* were susceptible; larvae of *Culex salinarius, Cx. quinquefasciatus, Cx. territans* and *Anopheles quadrimaculatus* were not susceptible (Clark and Fukuda, 1971).

Because *Oc. sollicitans* could not be reared in the laboratory, studies of OcsoNPV replication were undertaken in orally infected larvae of *Georgecraigius epactius* (formerly *Ochlerotatus epactius*). Thirty minutes after *Gc. epactius* larvae had been exposed to OcsoNPV, polyhedra were observed in the anterior midgut lumen. Some occlusion bodies showed signs of dissolution, due it was thought to the high pH of the midgut contents (Stiles *et al.*, 1983). Occlusion bodies of OcsoNPV gradually dissociated when placed in 0.03M NaOH (Federici and Anthony, 1972), of which the pH is ≈12.3. However, measurements of pH reported for the contents of different regions of the midgut of *Gc. epactius* larvae were: gastric caeca, pH 7.0; anterior midgut, pH 10.0; posterior midgut, pH 6.5–7.0 (Stiles and Paschke, 1980). One hour after exposure of *Gc. epactius* larvae to OcsoNPV OBs, free virions were present in the midgut lumen. Infection was limited to nuclei of the anterior midgut and, less often, the gastric caeca. It was not known how virions reached the midgut epithelium. The peritrophic membrane formed a discrete tube extending through the length of the midgut, and no virions were seen in the ectoperitrophic space. By 12 h post-infection (p.i.), nucleocapsids were present within nuclei of the anterior-midgut epithelium, and by 18 h p.i. the hypertrophied nuclei contained many nucleocapsids, some of which were acquiring envelopes. By 24 h, the nuclei contained nucleocapsids of usual width but unusual length, which approximated multiples of the prevalent 180 nm length (Stiles *et al.*, 1983).

(e) Uranotaenia sapphirina NPV (UrsaNPV)

In *Ur. sapphirina* larvae, virus development occurred only in nuclei of epithelial cells of the gastric caeca and posterior midgut, the visibly hypertrophied nuclei appearing as white nodules. No infections were seen in regenerative cells or in the cardia. UrsaNPV particles developed in one or other of budded virions or occluded virions. Infected nuclei contained a virogenic stroma, which first had a granular appearance and later, at its periphery, had large parallel arrays of nucleocapsids, where nucleocapsids of up to 400–500 nm length were adjacent to others of about 200 nm length (Figure 43.3A). The stroma matured into dense structures located throughout the nucleus which were involved in occlusion of virions. Vesicles that appeared within the nuclei were the precursors of nucleocapsid envelopes. Envelopment was initiated when nucleocapsids became attached to the membrane of vesicles, which surrounded them (Figure 43.3B). Budded virions formed when enveloped virions budded singly through the nuclear membranes, becoming enclosed in a vesicle formed of nuclear membrane (Figure 43.3B,C). The nuclear membranes soon disappeared. The mechanism by which

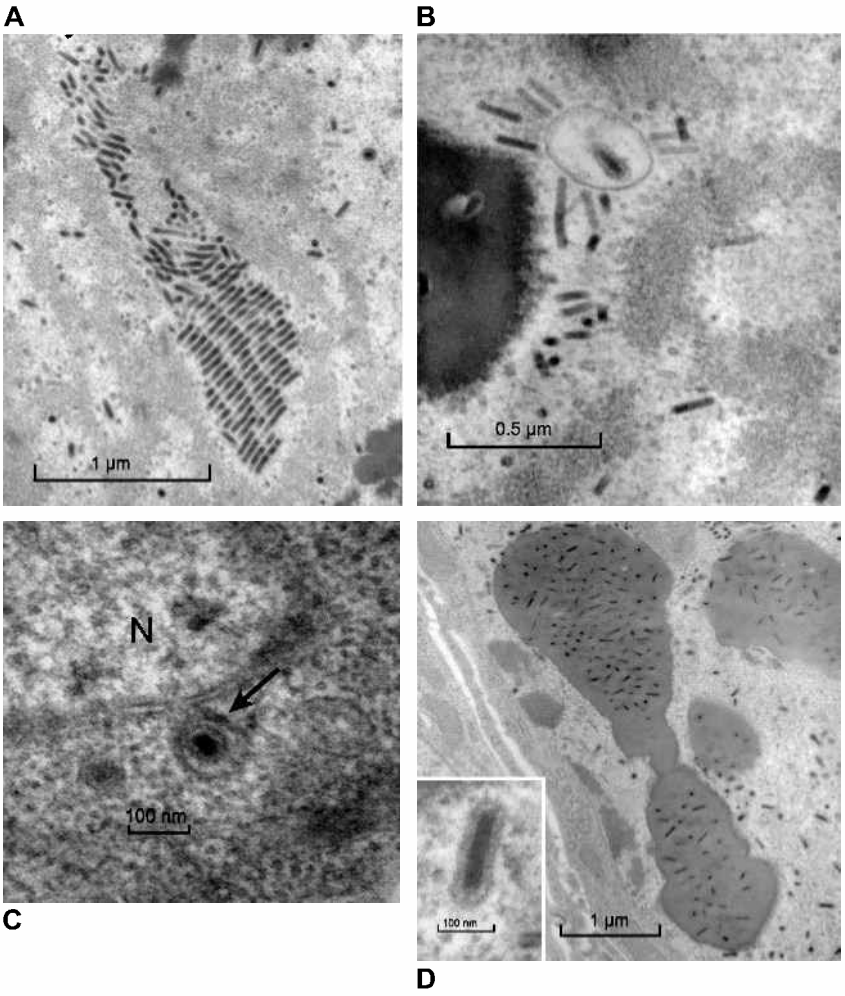

Figure 43.3 Transmission electron micrographs of Uranotaenia sapphirina NPV (UrsaNPV) in *Uranotaenia sapphirina* larvae. (Reproduced from Shapiro *et al.* (2004) with permission.) **A**. Parallel array of nucleocapsids in a virogenic stroma, showing short and long nucleocapsids. The short nucleocapsids are uniform in length; the combined length of two short ones is approximately that of a long nucleocapsid. **B**. Membranous envelope precursors surrounded by nucleocapsids, and showing one enveloped virion that has entered a vesicle. **C**. Budding of a virion through the nuclear envelope (black arrow). Note the presence of the viral envelope and acquisition of nuclear membranes; N, nucleus. **D**. Mature, dumbbell-shaped occlusion body with numerous virions; inset, a virion.

the virus disperses through the midgut epithelium is not known (Shapiro *et al.*, 2004). Other virions became occluded.

Within single aquatic habitats, the individual larvae of a susceptible species can be host to a nucleopolyhedrovirus (Baculoviridae), or a cypovirus (Reoviridae), or to both viruses, as described for populations of *Oc. sollicitans* in salt-marsh pools in Louisiana (Clark and Fukuda, 1971) and a population of *Ur. sapphirina* in a pond in Florida (Shapiro *et al.*, 2004). During 2001 and 2002, and principally during the months of October and November, larvae of *Ur. sapphirina* collected from a pond situated in pasture in Florida were variously infected with the nucleopolyhedrovirus UrsaNPV, or the cypovirus UsCPV-17, or with both viruses.

In the dually infected larvae, UrsaNPV was observed in the nuclei of some midgut epithelial cells and the cypovirus UsCPV-17 in the cytoplasm of others. The mean monthly rates of infection with UrsaNPV alone were 2.0–8.1% in 2001 and 8.3–14.7% in 2002. Those for dual infection were 2.0–7.1% in 2001, and 8.8–45.6% in 2002 (Table 43.4). The natural drying out of the pool from June to July 2002 did not prevent the reappearance of UrsaNPV in October. A small transient population of *Ur. lowii* larvae was also present in the pond in 2001, when the mean monthly infection rates with UrsaNPV did not exceed 1.4% and with UsCPV-17 did not exceed 2.0%. No dual infections were observed (Shapiro *et al.*, 2004).

Uranotaenia larvae collected from sites in which historically UrsaNPV had not been present were exposed in the laboratory to infected, wild-caught *Uranotaenia* spp. When kept in deionized water there was no transmission of the virus to *Ur. sapphirina* or *Ur. lowii* larvae, but in 10 mM $MgCl_2$ solution mean infection rates were recorded of 12.9% in *Ur. sapphirina* and 14.5% in *Ur. lowii*. Patent infections developed 2–4 days after exposure, and the larvae died on days 3–5. Second-instar larvae of *Stegomyia aegypti* and *Cx. quinquefasciatus* could be horizontally infected with UrsaNPV, but those of *An. quadrimaculatus* could not be (Shapiro *et al.*, 2004).

43.2.2 Family *Flaviviridae*

The family *Flaviviridae* consists of three genera, of which the genus *Flavivirus* is of particular importance because most species are arboviruses. For details of the genomic composition, classification and phylogenetic interrelationships of flaviviruses see Section 44.1.2.

Some nine unusual viruses of which the coding sequences are known to have the characteristics of insect-only viruses. They have the molecular characteristics of the genus *Flavivirus*, but share only a very low sequence identity with any of the accepted groups of *Flavivirus* species, while sharing some phenotypic characters with each other. All can replicate in one or other of the available mosquito cell lines, but certain of them cause cytopathic effects on other mosquito cell lines. They are unable to replicate in mammalian cell lines and do not have cytopathic effects on those cells. All were first isolated from either mosquito cell lines or wild adult mosquitoes, and as a group are currently referred to as 'insect-only flaviviruses'. Their nomenclature remains to be formalized, but one of them, Cell fusing agent virus (CFAV), is listed in Fauquet *et al.* (2005) as a 'Tentative Species in the genus *Flavivirus*'. The others are Kamiti River virus (KRV), Culex flavivirus (CxFV), Quang Binh virus (QBV), Nakiwogo virus (NAKV), nounané

Table 43.4 Seasonal rates of infection of *Uranotaenia sapphirina* larvae in a small pond in Florida with the nucleopolyhedrosis virus UrsaNPV only, the cypovirus UsCPV-17 only, or both viruses. (From Shapiro *et al.*, 2004.)

		UrsaNPV only	*UsCPV-17 only*	*Both viruses*	*Total larvae*
Year	*Month*	*% infected*	*% infected*	*% infected*	n
2001	October	4.2	10.8	3.2	1642
	November	8.1	9.4	7.1	721
	December	2.0	3.0	2.0	99
2002	April–September	0	0	0	266
	October	14.7	7.5	45.6	260
	November	8.3	12.5	8.8	970
	December	0	0	0	7

virus (NOUV), Stegomyia (Aedes) flavivirus (StFV), and Lammi virus (LAMV).

The first of these viruses to be discovered was isolated from a cell line derived from *St. aegypti* embryos, and was distinguishable only by its ability to cause *St. albopicta* cells to fuse and form syncytia, i.e. large cytoplasmic masses not divided into cells and containing many nuclei. It was named Cell fusing agent virus (CFAV), although it failed to cause cell fusion in *St. aegypti* cell lines or in three mammalian cell lines (Stollar and Thomas, 1975). No detectable serological cross-reactions had been found between CFAV and members of the genus *Flavivirus*, but its sequenced genome confirmed its affinities with that taxon (Cammisa-Parks *et al.*, 1992). CFAV was isolated from adult males and females in wild populations of *St. aegypti, St. albopicta*, and *Culex* spp. in Puerto Rico (Cook *et al.*, 2006).

Kamiti River virus (KRV) was isolated from adults of *Neomelaniconion mcintoshi* reared from larvae and pupae collected from pools in a dambo habitat in central Kenya (Crabtree *et al.*, 2003; Sang *et al.*, 2003). [The authors erroneously named this mosquito *Aedes macintoshi*, but that is an Australian species, now named *Ochlerotatus macintoshi*.] Culex flavivirus (CxFV) was isolated in Japan from wild populations of *Cx. pipiens* and other *Culex* species (Hoshino *et al.*, 2007). Phylogenetic analyses of isolates of CxFV from mosquitoes collected in different regions grouped them in four clades: one clade with isolates from Central America and Trinidad, and a group of three clades with isolates from Indonesia, Japan and the United States (Blitvich *et al.*, 2009; Kim *et al.*, 2009). Quang Binh virus was isolated from a pool of *Culex tritaeniorhynchus* collected in Quang Binh, Vietnam (Crabtree *et al.*, 2009). Nounané virus (NOUV) was isolated from adult *Uranotaenia mashonaensis* collected in primary rain forest of the Taï National Park in Côte d'Ivoire. The name, correctly, is nounané virus. The initial letter is not capitalized because it is not a proper noun but rather, in the local Oubi language, means 'having one's own way' (Junglen *et al.*, 2009). Nakiwogo virus (NAKV) was isolated from *Mansonia africana nigerrima* in Uganda, and is named after the location where the mosquitoes were trapped (Cook *et al.*, 2009). Stegomyia flavivirus (StFV), then named Aedes flavivirus, was isolated from *Stegomyia albopicta* and a related species in Japan. It replicated in a mosquito cell line, producing a mild cytopathic effect (Hoshino *et al.*, 2009). A sequence that closely resembles KRV, named 'cell silent agent' (CSA), is integrated into the genome of *St. albopicta* (Crochu *et al.*, 2004). The phylogenetic relationships of insect-only viruses to other species of the genus *Flavivirus* are discussed in Section 44.1.2.b.

Sequences from ssRNA flavivirus genomes have been found in the dsDNA form integrated into the genomes of certain aedine mosquitoes. Sequencing DNA extracts of cultured C6/36 *St. aegypti* cells (then named '*Aedes aegypti*' cells) revealed an ORF (open reading frame) of 1557 amino acids that was closely related to the ssRNA *NS1–NS4* genes of Cell fusing agent virus and Kamiti River virus. The corresponding mRNAs were also identified. Integrated sequences homologous to three other flavivirus genes were also identified, and overall approximately two-thirds of a flavivirus-like genome were characterized within the extracted DNA. In cultured A20 *St. aegypti* cells, a 492 amino acid ORF related to polymerases of CFAV and KRV was identified. These findings suggested past infections with unknown viruses. Similar flavivirus-related, integrated DNA sequences were detected in wild-caught *St. albopicta* and *St. aegypti*, so indicating a novel mode of horizontal gene transfer between eukaryotic cells, and a mechanism by which genetic diversity might be generated (Crochu *et al.*, 2004).

Lammi virus (LAMV) was isolated from mass collections of mosquito larvae from two locations in Finland, one being Lammi, probably from *Aedes cinereus*. In a phylogenetic tree it was a sister lineage with nounané virus (Huhtamo *et al.*, 2009). Calbertado virus (CALBOV) was isolated from *Culex tarsalis* in western Canada, Colorado and California (Tyler *et al.*, 2011).

Little is known of the pathogen–host interactions of insect-only flaviviruses, or of their mode of perpetuation. The detection of insect-only viruses in the aquatic stages or adult males of

wild-caught mosquitoes – as for CFAV (Cook *et al.*, 2006), CxAV (Hoshino *et al.*, 2007), StFV (Hoshino *et al.*, 2009), and KRV (see above) – is indicative of vertical transmission. Egg rafts of *Cx. pipiens* were collected in the field in Iowa and reared to produce adults, taken to be the parental (P) generation in experiments that followed. From the 26 fertile (F_1) egg rafts that they produced, 18 proved positive for CxFV, a vertical transmission rate of 69.2%. From each CxFV-positive F_1 female, 30 F_2 female progeny were tested, a total of 540. Of those, 526 proved to be CxFV-positive, a filial infection rate of 97.4%. These results were taken to suggest that perpetuation of CxFV might be possible through vertical transmission alone (Saiyasombat *et al.*, 2011).

43.2.3 Family *Iridoviridae*

(a) Characteristics

Genome. A single molecule of linear dsDNA. The genome is terminally redundant; i.e. each virus particle contains a complete genome plus about 10% redundant DNA, and it is circularly permuted, viz. the terminal sequences are different for each particle in a population. Size, 135 to 303 kbp. The genomes include extensive repeat sequences with 20% of repetitive DNA observed for Invertebrate iridescent virus 3 (IIV-3). *Virion*. Of exceptionally large size, the virions range from 120 to 200 nm in diameter and are of icosahedral symmetry. The core is a nucleoprotein filament surrounded by a lipid membrane containing transmembrane proteins. The capsid is formed of many identical protein capsomers, approximately 6–7 nm in diameter and 7–13 nm in height, arranged in trimers and pentamers. The number of capsomers depends on virion size. Virions that bud out of host cells may acquire an outer envelope. Both the naked and enveloped virions are infectious. *Replication*. Iridoviruses replicate in most insect tissues, but especially the fat body, haemocytes and epidermis. Infection is rarely seen in midgut epithelial cells, so the midgut is unlikely to be the place of entry (Fauquet *et al.*, 2005; Williams and Ward, 2010). *Hosts*. Invertebrates or poikilothermic vertebrates, varying with the genus (King *et al.*, 1994; Williams, 1996, 1998; Fauquet *et al.*, 2005).

The genome of a field isolate of Invertebrate iridescent virus type 3 from *Oc. taeniorhynchus* larvae comprised 190 kbp, of which *c.* 20% were repetitive DNA, with repeats localized in 15 apparently non-coding regions. Of 112 of the 126 predicted genes, 27 genes had homologues in all other sequenced iridescent viruses; 52 genes were present in the type species of the genus *Iridovirus* but not in vertebrate-infecting iridescent viruses; and 33 genes lacked homologues in the other sequenced iridescent viruses. These findings possibly reflect distinct evolutionary histories for the vertebrate and invertebrate iridescent viruses (Delhon *et al.*, 2006).

(b) Classification

Based on particle size, host range, DNA cross-hybridization, the presence of a methyl transferase and the major capsid protein sequence, the family *Iridoviridae* (00.036) is classified into five genera, of which two, *Iridovirus* (00.036.0.01) and *Chloriridovirus* (00.036.0.02), infect invertebrates, particularly insects, and three infect poikilothermic vertebrates. The species of all five genera are referred to as 'iridoviruses', although the members of only three cause iridescence in tissues of their hosts. The genus *Iridovirus* comprises two accepted species and 11 tentative species. The two accepted species are *Invertebrate iridescent virus 1* (IIV-1), synonym Tipula iridescent virus, and *Invertebrate iridescent virus 6* (IIV-6), synonym Chilo iridescent virus. The 11 tentative species have been isolated from insects and terrestrial isopods. Particle diameter is 120–130 nm. The genus *Chloriridovirus* contains just one species, *Invertebrate iridescent virus 3* (IIV-3), which has the synonyms Stegomyia taeniorhynchus iridescent virus and Mosquito iridescent virus, and which is known only from culicids. Distinguishing features of the genus *Chloriridovirus* include: a particle diameter of approximately 180 nm; trimers and pentamers larger than in the genus *Iridovirus*, with probably 14 capsomers to each edge of the trimer; and a virion M_r of approximately 2.49–2.75 $\times 10^6$ (Fauquet *et al.*, 2005).

The strain of iridescent virus selected for description as the type species of the genus *Chloriridovirus* was originally isolated from wild larvae of *Oc. taeniorhynchus* caught near Vero Beach, Florida (Clark *et al.*, 1965); it was then given the name *Invertebrate iridescent virus 3* (abbreviation IIV-3), and the synonyms Aedes taeniorhynchus iridescent virus and Mosquito iridescent virus. It has also been called Vero Beach IV and regular strain (Murphy *et al.*, 1995; Williams, 1996, 1998). The genome of the mosquito-infecting IIV-3 resembled the genomes of other iridescent viruses in size, DNA composition and gene complement. Among the completely sequenced iridescent viruses, IIV-3 most closely resembled IIV-6, but it had novel genomic features that indicated a distant relationship to other iridescent virus genera and confirmed its unique position within the family Iridoviridae (Delhon *et al.*, 2006).

(c) *Iridescence*

In species of the genera *Iridovirus*, *Chloriridovirus* and *Ranavirus*, the virus particles, when present in high density within host cells, assemble in paracrystalline arrays (Figure 43.4A), and when beams of incident white light fall on these arrays, the emitted light is iridescent. Iridescence can be defined as a rainbow-like play of colour caused by a differential refraction of light waves. The reflected beams are seen as single colours, which tend to change as the angle of view changes. The physical basis of the iridescence of the Mosquito iridescent virus (IIV-3) has not been examined, but that of *Invertebrate iridescent virus 1* (synonym Tipula iridescent virus, TIV) has been, and those findings merit description here.

Particles of TIV released from disintegrating cadavers of infected *Tipula paludosa* larvae were separated from cellular debris by centrifugation, yielding pellets with an iridescent turquoise appearance. When embedded, sectioned and examined by transmission electron microscopy, each pellet was seen to consist of regions of crystallinity averaging 5–10 μm diameter. Isolated virus particles that were air dried or frozen dried before examination by scanning electron microscopy were ~130 nm in diameter and icosahedral in shape, all their facets being equilateral triangles (Williams and Smith, 1957, 1958). The crystallites were examined further using the principles of X-ray crystallography, a technique described here by reference to studies of mineral crystals.

X-ray crystallography exploits the similarity between the dimensions of atoms, which are of the order of 0.1 nm, and the wavelengths of X-rays (10^{-3} to 10 nm) – which include a band of wavelengths of the same order of magnitude. Finding that exposure of crystalline solids to X-rays of certain wavelengths and angles of incidence produced intense peaks of emitted radiation, Lawrence Bragg modelled the crystal as a regular arrangement of atoms set in discrete parallel planes separated by a constant distance d. This is now described as a lattice structure. Bragg suggested that, instead of considering that each atom acts as a scattering point for X-rays (as it does), for purposes of description the planes of atoms that make up lattices should be regarded as reflecting units, as for example in the NaCl crystal which consists of a cubic array of sodium and chloride ions (Figure 43.4B).

If X-rays of wavelength λ are incident on such a crystal, diffracted rays of maximum intensity occur only in those directions in which constructive interference takes place between the rays scattered by successive lattices. (Constructive interference occurs when the peaks and troughs of two waves that are of the same frequency and that are in phase line up. With two waves of amplitudes A_1 and A_2, the resultant wave A will have the amplitude $A = A_1 + A_2$.) In Figure 43.4B, the path difference introduced between ray 1 and ray 2 will be AB + BC, and, if θ is the glancing angle of the X-rays, then the path difference = $2d \sin \theta$, where d is the interplanar spacing. The Bragg equation, which concerns the relationship between X-ray wavelength, angle of incidence and the spacing between lattices, indicates that reflection of X-rays from a lattice plane occurs at an angle of θ, i.e. the angle between the plane and the incident beam of X-rays, and satisfies the equation

$$n\lambda = 2d \sin \theta \qquad (43.1)$$

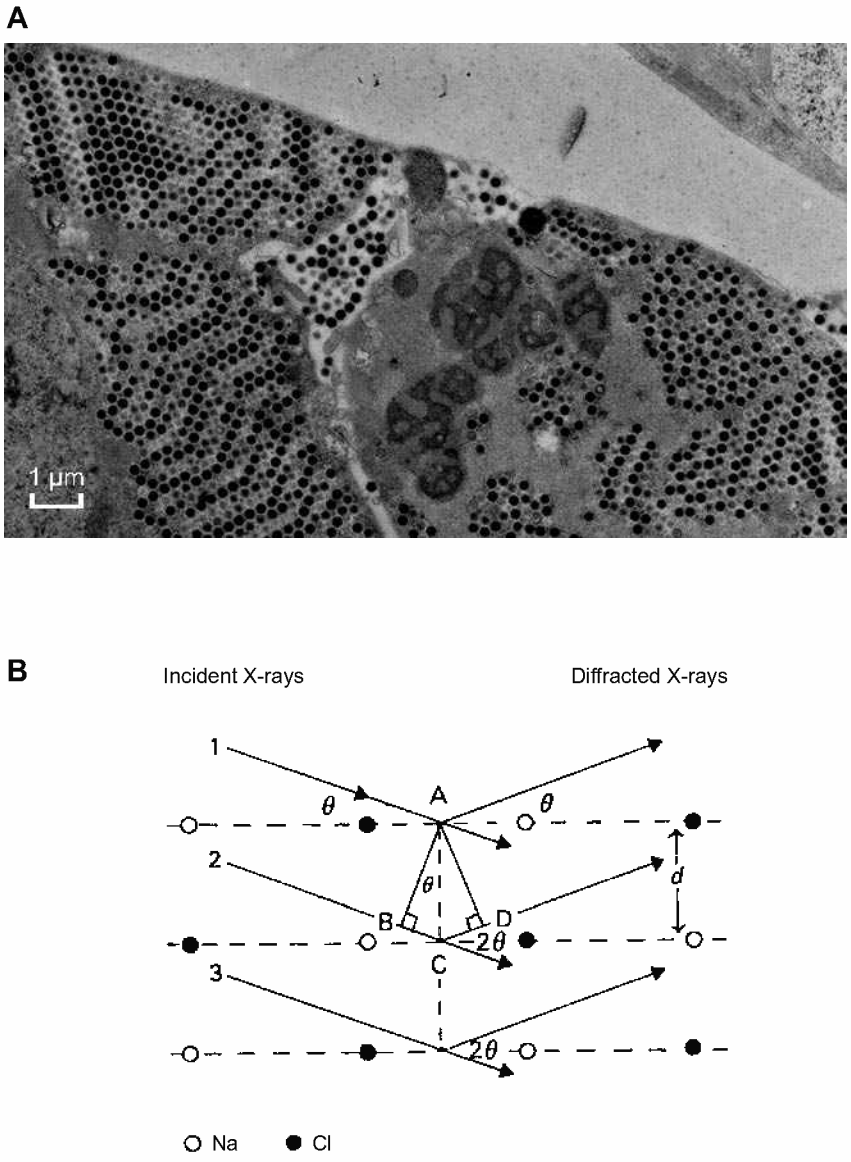

Figure 43.4 A Section through fat body cells of a larva of *Ochlerotatus taeniorhynchus* infected with *Invertebrate iridescent virus 3*, showing paracrystalline arrays of virions within the cytoplasm. Scale bar, 1 µm. (Micrograph by courtesy of Ms Susan White.) B Representation of the scattering of X-rays from the lattice planes, distance d apart, in a sodium chloride crystal. (Modified from Duncan, 1990.) Three rays with identical wavelength and phase, approach the crystal and are scattered off three planes. Rays 1 and 2 are reflected from identical planes, ray 2 traverses an extra length of $2d \sin \theta$, where θ is the angle of incidence and also the scattering angle. In order for waves to interfere constructively, the difference in travel path (B C D) must equal one wavelength or a multiple of one wavelength.

where n is an integer number of the wavelength, and θ is the angle between the incident ray and the scattering planes. The angle between the incident and reflected rays is 2θ (Duncan, 1990).

Beams of X-rays could not be used to examine the structure of the crystals of Tipula iridescent virus (TIR) because of the relatively massive distance between the lattice planes, but beams of visible light of appropriate wavelength could be used for that purpose. Crystals of TIR were prepared by transfer of a pellet of virus particles and a drop of supernatant into a rectangular Perspex cell of 4 × 2 × 0.7 mm. Over a period of days and in the weak gravitational field, large crystals formed from the microcrystalline pellets, with thin plates, or crystal planes, lying parallel to the surface of the Perspex cell. When exposed to monochromatic light, the reflected light was green or turquoise at small angles of incidence, and blue or violet at larger angles. These were hydrated crystals, similar to those that formed within host cells. The virus concentration, calculated from the measured refractive index, was 17.4 ± 4% w/v. Within crystallites, the virus particles were packed in a face-centred cubic array (i.e. having an atom located at the centre of each face of the unit cell), with an inter-particle spacing of 250 nm – a distance nearly twice the diameter of a frozen-dried particle and approaching the wavelengths of the visible spectrum. It was concluded that the hydrated virus particles in a crystal are not in contact but are separated by water at distances of ~50 nm. The crystals are separated owing to repulsive Coulombic interactions between electrically charged particles in the aqueous environment.

For any given wavelength, refraction occurred only under specular conditions (when the angle of incidence and angle of reflection were equal with respect to the face of the rectangular cell). The angle of incidence which gave reflection was a direct function of the wavelength, and it was confirmed that rotation of the cell in its own plane made no difference. It was concluded, therefore, that the equivalent of Bragg reflection was taking place from crystal planes parallel to the cell face. Crystallites that lie at an appropriate orientation to an incident beam of white light will reflect a particular wavelength, which depends on inter-particle spacing within the crystallite. The wavelength selectively reflected is proportional to the sine of the glancing angle and to the distance between successive reflecting planes in the crystal (Klug*, Franklin‡ and Humphreys-Owen†, 1955). As for other, comparable, cases in nature, the brilliant iridescence can be attributed to the diffraction and constructive interference of waves of visible light, of appropriate wavelength, which satisfy Bragg's law in a manner analogous to the scattering of X-rays in a crystalline solid.

(d) Biology

Invertebrate iridescent virus 3 (IIV-3) has been isolated from wild larvae of five *Ochlerotatus* species (*Ochlerotatus annulipes*, *Ochlerotatus cantans*, *Ochlerotatus fulvus*, *Oc. stimulans*, *Oc. taeniorhynchus*), *Am. vexans*, *Ps. ferox*, *Culiseta. annulata* and *Cx. territans* (Weiser, 1965; Chapman *et al.*, 1966; Anderson, 1970; Rivers and Service, 1971; Buchatsky, 1977). Apparent infection rates of wild insect populations with iridescent viruses are very low; often only one individual among many thousands is obviously infected (King *et al.*, 1994; Williams, 1996). In a habitat of *Oc. taeniorhynchus* larvae which was flooded six times between July and September, the infection rate rose from

*, In 1982, the Nobel Prize in Chemistry was awarded to Aaron Klug 'for his development of crystallographic electron microscopy and his structural elucidation of biologically important nucleic acid-protein complexes'.

‡, From her X-ray photographs and by applying the methods of Paterson function analysis, Rosalind E. Franklin showed that the structure of the two hydrated forms of ribonucleic acid was best accounted for by a double spiral of nucleotides, in which the phosphorus atoms lay on the outside. Without her knowledge, Franklin's diffraction photograph of the B form of DNA was shown to a visitor, providing compelling evidence for the helical structure of DNA. Rosalind Franklin died on 15 April 1958, aged 37.

†, From analysis of the intensity of light scattered by crystals of NaCl as a function of wavelengths into the ultraviolet, S.P.F. Humphreys-Owen (1955) attributed the greatest part of the scattering to a submicroscopic block structure of the crystals of the order of 150 nm.

0.001% to 0.5% during the 3-month period (Chapman *et al.*, 1966). In snowmelt pools containing infected larvae of *Oc. annulipes* and *Oc. cantans*, not more than 1% of larvae were infected. Pools containing infected larvae might be separated by only 2 m from pools with uninfected larvae. The infected larvae appeared to die before pupation (Weiser, 1965).

In the laboratory, *Oc. taeniorhynchus* larvae became infected with IIV-3 after feeding on a macerate of infected larvae, and high rates of transmission could occur in densely crowded larval cultures. Infections that started during the early instars did not inhibit growth or development, but the larvae died during the fourth instar. Exposure of early fourth-instar larvae to low densities of live infected fourth-instar larvae resulted in transmission. Iridescent virus from *Oc. taeniorhynchus* infected larvae of that species and of *Oc. sollicitans* but not of *Ps. ferox* or *Am. vexans*; iridescent virus from *Ps. ferox* infected larvae of that species but not of *Oc. taeniorhynchus* or *Am. vexans* (Linley and Nielsen, 1968a,b; Woodard and Chapman, 1968; Matta and Lowe, 1970).

Only low rates of infection occurred in the laboratory upon exposure of *Oc. taeniorhychus* larvae to macerated larvae infected with IIV-3 in the laboratory, the highest (32%) being with second-instar larvae (Woodard and Chapman, 1968). After exposure to a suspension of *c.* 1 mg virus per ml overnight, rates of infection ranged between 4.2% and 19.7%, with a mean of 8.1%. Electron-microscopic examination revealed that first- and second-instar *Oc. taeniorhynchus* larvae ingested large amounts of IIV-3. Virus particles were present in cells of the foregut, but almost entirely absent from the midgut, where they were rapidly degraded. No particles were seen outside the peritrophic membrane (Stoltz and Summers, 1971). Higher infection rates could be achieved by feeding larvae with a mixture of virus particles and silicon carbide fibres, suggesting that infection occurs when the virus invades through breaks in the cuticle or peritrophic membrane (Undeen and Fukuda, 1994). In *Oc. taeniorhynchus* infected with IIV-3, the fat body was the most affected tissue, paracrystalline arrays of virus particles replacing the cytoplasm of each cell, while the nuclei were unaffected. Viral particles were present at lower densities in cells of the epidermis, tracheal epithelium, imaginal discs and ovaries; the alimentary canal was mostly free of infection, only the oesophageal cells containing viral particles. Larvae became iridescent during the fourth instar, appearing yellow to green, orange, pink or blue; sometimes, at a later stage of infection, they appeared milky white (Woodard and Chapman, 1968; Hall and Anthony, 1971).

Vertical transmission of invertebrate iridescent virus 3 in *Oc. taeniorhynchus* was demonstrated when larvae that were exposed to viral particles during the third or fourth instar survived to adulthood and an infection appeared in their progeny (Woodard and Chapman, 1968). Vertical transmission was investigated further by exposing to infection larvae that were just moulting to the fourth instar; adult females derived from these were fed and the eggs that they laid were surface sterilized. An infection rate of 12.8% was recorded in the second-generation larvae. This was transovarian transmission (Section 41.2.3.b). Larvae of *Oc. taeniorhynchus* that were orally infected early in development died during the fourth instar. Virions dispersed from the cadavers could infect younger larvae present at the time, but were unlikely to survive to infect later generations because they became non-infective soon after liberation from the host tissues, i.e. in under 1 day if exposed to soil, after 5 days in fresh water, and after 20–30 days in salt water. It was postulated that transovarian transmission produces infected larvae, which die in the fourth instar. Healthy fourth-instar larvae that feed on the cadavers develop to infected adults, and the transmission cycle is completed when the adult females lay infected eggs (Linley and Nielsen, 1968a,b).

Under laboratory conditions, larvae of *St. aegypti* (an experimental host) could be infected with Invertebrate iridescent virus 6 (IIV-6), the type species of *Iridovirus*. Exposure of batches of *St. aegypti* larvae to different amounts of IIV-6 led to 0–1.5% of easily observable (patent) infections and to 0–20% of covert infections. Larvae with patent infections developed an iridescent lilac-blue

colour due to assemblages of virus particles in crystalline arrays in the cytoplasm of infected cells, and these infections were invariably fatal. Covert infections, detected by amplification of viral DNA coupled with bioassays, were sublethal, prolonging the aquatic stages and reducing wing length, fecundity and longevity of the adult females (Marina *et al.*, 1999, 2003).

43.2.4 Family *Nodaviridae*

Characteristics. The genome consists of two molecules of positive-sense ssRNA; both molecules are required for infectivity and both are encapsidated in the same virus particle. Size, 4–5 kb. *Virion*. Roughly spherical, with isometric symmetry, 25–37 nm in diameter and non-enveloped. Nodaviruses contain 60 subunits of three similarly folded capsid proteins. *Replication*. Transcription, genome replication and virion assembly occur in the cytoplasm of infected cells.

The family *Nodaviridae* (00.045) consists of two genera, *Alphanodavirus* (00.045.0.01) and *Betanodavirus* (00.045.0.02), which are distinguished from one another by particle size (slightly larger in alphanodaviruses) and the occurrence of empty particles (seen in some preparations of betanodaviruses). The genus *Alphanodavirus* comprises five named species and three tentative species, all of which were isolated from insects. *Nodamura virus* (NoV) (00.045.0.01.001), the type species of the family, was isolated from mosquitoes. It appears unique among nodaviruses in also naturally infecting pigs and cattle. Betanodaviruses were isolated from fish (Fauquet *et al.*, 2005). Nodamura virus differed from the known arboviruses in not being inactivated by diethyl ether or chloroform (Scherer and Hurlbut, 1967).

Nodamura virus has been isolated only once, which was from a pool of 500 female *Cx. tritaeniorhynchus* captured on 23 July 1956 in a trap baited with two pigs at the Sagiyama heronry, Nodamura village (now the city Nodashi), near Tokyo. Many of the females had engorged on the pigs, which showed no overt signs of illness, and the one pig from which plasma was taken for serological tests did not develop anti-NoV neutralizing antibody within 22 days after the infected mosquitoes were collected. There was no evidence that the pigs had been the source of the virus. The collection of NoV-infected *Cx. tritaeniorhynchus* took place during the peak of the *Cx. tritaeniorhynchus* population density (Scherer and Hurlbut, 1967).

Inapparent infections in pigs occurred in three locations near Tokyo during 1956 and 1957. The pigs, mostly about 6 months of age, contained anti-Nov neutralizing antibodies in their plasma when bled in August and early September. The farmed pigs were bitten frequently and by large numbers of *Cx. taeniorhynchus*. Serological tests on cattle in the Tokyo area during September and November 1959 revealed anti-NoV neutralizing antibodies, suggesting that the cattle had carried natural infections with Nodamura virus. Of 54 nestling or juvenile ardeid birds of three species sampled at the Sagiyama and Shinhama heronries near Tokyo during 1956 and 1957, only two out of 27 Black crowned night herons (*Nycticorax nycticorax*) were infected.

In the laboratory, *Cx. taeniorhynchus* did not readily feed on infected suckling mice, but *St. aegypti* readily engorged on viraemic mice and transmitted NoV to healthy mice. *Culex tarsalis* also was an effective vector. When inoculated into suckling mice, NoV caused necrosis of spinal cord neurons and degeneration of limb skeletal muscles and paravertebral muscles, causing hind limb paralysis and death within 5 to 15 days (Scherer and Hurlbut, 1967; Scherer *et al.*, 1968).

There is insufficient evidence to establish whether Nodamura virus is an arbovirus. Karabatsos (1985) classed it as 'probably not an arbovirus', but, on the grounds that it was isolated from mosquitoes, and was capable of replication in mosquitoes and of biological transmission by mosquitoes, Scherer *et al.* (1968) considered it to be an arbovirus.

43.2.5 Family *Parvoviridae*

(a) Characteristics and classification

Genome. An unsegmented, linear molecule of ssDNA, 4–6 kb in length; variable amounts of the (+) or (–) strand are encapsidated. They are

characterized by the self-priming hairpin termini of their genomes to which the protein NS1 binds to initiate viral DNA replication. The genome contains two sets of open reading frames, for structural and non-structural proteins. In vertebrate-infecting parvoviruses, the coding sequences for non-structural (*NS*) and capsid (*VP*) genes are on the same strand (monosense organization), whereas in some invertebrate-infecting arboviruses the *NS* and *VP* genes are located in the 5' half of each strand (ambisense organization). *Replication*. Parvoviruses replicate in the host-cell nucleus, and most multiply in all tissues. *Virions*. Small, 18–26 nm in diameter, non-enveloped; the capsids exhibit icosahedral symmetry. The particles are composed of 60 copies of the region of the capsid protein that is common to all forms of the structural polypeptides. None of the proteins is glycosylated. Entry of virus into the host cell is by receptor mediated endocytosis, and is blocked by antagonists of vacuolar ATPase (Fauquet *et al.*, 2005; Baquerizo-Audiot *et al.*, 2009).

The family *Parvoviridae* (00.050) is separated into two subfamilies – the *Parvovirinae* (00.050.1) which infect mammals, and the *Densovirinae* (00.050.2) which infect arthropods. The *Eighth Report of the ICTV* (Fauquet *et al.*, 2005) lists four genera of densoviruses (DNVs), *Densovirus*, *Pefudensovirus*, *Iteravirus* and *Brevidensovirus*, but since then other genera have been recognized by the ICTV or proposed. The host range of densoviruses is limited to certain insect orders and in a small number of species to shrimps (Crustacea).

Species of ***Brevidensovirus*** have a 4 kb genome with terminal hairpins but no inverted terminal repeats. Populations of virions encapsidate positive and negative strands, but 85% of strands are of negative polarity. Five species of *Brevidensovirus* with formal ICTV recognition are listed in Table 43.2. The type species of the genus *Brevidensovirus* is *Stegomyia aegypti densovirus* (SaeDNV), first isolated from laboratory colonies in which it infected larvae, pupae and adults (Buchatsky, 1989). *Stegomyia albopicta densovirus* (SalDNV) is known as three distinct viruses isolated from C6/36 cell lines, which are referred to here as SalDNV-1 (Jousset *et al.*, 1993; Boublik *et al.*, 1994), SalDNV-2 (Chen *et al.*, 2004) and SalDNV-3 (Paterson *et al.*, 2005). In three trees from cladistic analyses based on *NS1*, *NS2* and *VP* nucleotide sequences, the three forms of SalDNV showed as separate lineages (Zhai *et al.*, 2008). *Anopheles gambiae NDV* was first isolated by Ren *et al.* (2008). *Culex pipiens pallens* is a subspecies of *Cx. pipiens* which is indigenous to the Oriental Region. *Culex pipiens pallens* DNV (CppDNV) was isolated from adult female *Cx. pipiens pallens* wild caught in China (Zhai *et al.*, 2008). *Haemagogus equinus DNV* (HgDNV) was isolated from persistently infected *Hg. equinus* cell lines (O'Neill *et al.*, 1995). The one other known species of *Brevidensovirus* is *Penaeus stylirostris DNV* (correctly *Litopenaeus stylirostris*), from the Pacific blue shrimp (Fauquet *et al.*, 2005),.

A virus that had been isolated from a laboratory colony of *Cx. pipiens pipiens* was named *Culex pipiens DNV* (CpDNV), and was found to share antigenic properties and size and sequence properties with a species of *Densovirus* which had a lepidopteran host (Jousset *et al.*, 2000). From cladistic analyses of viruses from all four genera of Densovirinae and also of CpDNV, two neighbour-joining phylogenetic trees were produced based on NS1 and VP1 proteins. In both trees, the CpDNV lineage was situated distant from the *Brevidensovirus* clone and was a sister group to *Densovirus*. The CpDNV genome shared the main characteristics of viruses belonging to the genus *Densovirus* (all with lepidopteran hosts): an ambisense organization, the presence of inverted terminal repeats (ITRs), and significant identities at both capsid and *NS* gene levels. However, CpDNV deviated from that model in at least five genomic domains: (i) the terminal hairpin of the ITR; (ii) the capsid VP proteins; (iii) the ORFs of the *NS1* and *NS2* coding sequences; (iv) the number of promoters controlling the expression of *NS* genes; and (v) the location of the TATA boxes. Baquerizo-Audiot *et al.* (2009) concluded that, taken together, these structural differences strongly suggest that CpDNV differs sufficiently from other densoviruses in both its replication and expression strategies to be considered as the prototype of a possible new genus within the subfamily *Densovirinae*. A subsequent

proposal for a new genus, ***Cupidensovirus***, with CpDNV as prototype, was accepted by the ICTV.

(c) Biology

An inherent problem in laboratories that maintain a number of cell lines is the risk of cross-contamination with cells. Cross-contamination with viruses is an equal risk. In an investigation in which 11 cell lines from nine mosquito species were screened with primers designed to amplify fragments from SaeDNV and SalDNV, positive PCR amplification of fragments was obtained with four of the cell lines. Two of the isolates were new densoviruses. Of the different clones, those from a Stegomyia aegypti DNV cell line and a Culex theileri DNV cell line appeared to be identical with St. albopicta DNV originally isolated from C6/36 *St. albopicta* cells. The *Cx. theileri* cell line was initially established using filtered spent media from C6/36 *St. albopicta* cell lines – significantly, the source from which SalNPV was first cultured (O'Neill *et al.*, 1995).

A full-length clone of Stegomyia aegypti DNV was infectious when transfected into cultured mosquito cells. Developed into an expression and transducing vector, it could transduce genes of interest into live mosquitoes. In one application of this procedure, recombinant transducing virus expressing green fluorescent protein gene (GFP) was used to detect viral infection of *St. aegypti* larvae and pupae. Expression of GFP was detected in larvae and pupae as soon as 48 h post-infection. Midgut, hindgut and Malpighian tubule cells expressed GFP soon after transduction, but the anal papillae were the most commonly infected organ (Afanasiev *et al.*, 1999).

When newly hatched *St. aegypti* larvae were exposed to transducing particles, the points of infection could be identified by the sites of GFP expression. Among larvae showing a single site, the anal papillae provided that site predominantly. Many fewer infections were apparent at other locations, mostly either a single cell at the base of an anal papilla or a bristle cell elsewhere. Among larvae that showed more than a single site of infection, the anal papillae were again predominant. Cells of the alimentary canal were never the primary site of infection. Dissemination from infected anal papillae to fat body cells occurred in 60% of larvae after an average of 2 days. Usually, this was followed by infection of muscle fibres and other tissues. Most infected larvae died 2–3 days after virus dissemination. In over 50% of larvae with infected anal papillae, the papillae quickly shrank and became detached. About one-third of the infected larvae that lost their anal papillae showed no further evidence of GFP expression and developed normally, while two-thirds remained infected (Ward *et al.*, 2001).

Densoviruses multiply in most tissues of the host, and the name reflects the accumulation of virions in dense intranuclear masses. In hosts infected with Stegomyia aegypti DNV, the most obvious pathogenic changes are in the cells of the fat body, in which the nuclei come to contain densely packed virions and grow to 2–3 times the normal size. Later, many virus particles appear in paracrystalline arrays in the cell cytoplasm, and release of virions appears to be a consequence of cell destruction (Lebedeva *et al.*, 1973; Lebedinets *et al.*, 1978; Buchatsky and Raikova, 1979; Buchatsky, 1989). Larvae of all instars are susceptible to infection. When early-instar larvae become infected, the effects become apparent in the fourth-instar larvae and pupae. Infected larvae lose their mobility and hang from the water surface; infected pupae sink to the bottom. When late-instar larvae are infected, the effects are seen in the pupae and adults (Buchatsky, 1989). In the laboratory, horizontal transmission of Stegomyia aegypti DNV through ingestion of virions has been demonstrated in species of *Aedimorphus*, *Ochlerotatus*, *Stegomyia*, *Culex* and *Culiseta*. That surface sterilization of eggs did not prevent transmission of virus to the next generation was evidence of vertical transmission (Lebedinets *et al.*, 1978; Buchatsky, 1989; Lebedinets and Kononko, 1989). Stegomyia aegypti DNV had no pathogenic effects on mice, rats or chick embryos (Lebedinets *et al.*, 1976).

When *St. aegypti* larvae were infected with Stegomyia albopicta DNV, the fat body was the

first tissue to be visibly affected; later, dense intranuclear accumulations of particles appeared in most larval tissues, but not in midgut cells. Exposure of first-instar larvae to water in which infected larvae had been reared resulted in 32% mortality, and exposure to macerate of infected larvae led to 71–87% mortality; deaths occurred principally among the fourth-instar larvae or pupae. Exposure of third-instar larvae to infection resulted in 65% mortality; although 90% of surviving adults were infected, the females could lay eggs (Barreau *et al.*, 1996). Vertical transmission of SalDNV was demonstrated in *St. aegypti*. Among larvae from moderately and heavily infected mothers the filial infection rates were 85% and 90%, respectively; however, in the F_1 adults, the infection rates had fallen to 0% and 7.4%, respectively. A venereal transmission rate of 2.2% was measured when a batch of uninfected females was caged with a similar number of males, of which 90% were infected (Barreau *et al.*, 1997).

43.2.6 Family *Poxviridae*

Genome. A single, linear molecule of dsDNA, with covalently closed termini. Size, 130–375 kbp. *Virions*. Larger than those of other animal viruses and discernible by light microscopy. Somewhat pleomorphic; mostly either (i) brick-shaped, 220–450 nm long, and with a lipoprotein surface membrane displaying tubular or globular units, or (ii) ovoid, 250–300 nm long, and with a surface membrane possessing a regular spiral filament. The surface membrane encloses a core that contains the genomic DNA and proteins organized in a nucleoprotein complex. This virion form is described as an 'intracellular mature virus'; some of these particles may be wrapped by an additional double layer of intracellular membrane to form 'intracellular enveloped virus'. *Replication*. Transcription and genome replication occur predominantly but not exclusively within the cytoplasm of host cells. *Hosts*. Insects or vertebrates according to genus.

The family *Poxviridae* (00.058) comprises two subfamilies: the *Chordopoxvirinae* (00.058.1) with eight genera, infecting vertebrates; and the *Entomopoxvirinae* (00.058.2) with three genera, infecting insects. The viruses of the two subfamilies are similar in structure and have many molecular similarities. The most striking structural difference between them is the formation by species of the subfamily *Entomopoxvirinae* of virus-coded, proteinaceous occlusion bodies (or spheroids), which contain mature virus particles embedded in a paracrystalline matrix (King *et al.*, 1998; Fauquet *et al.*, 2005).

Viruses of the subfamily *Chordopoxvirinae* are transmitted between their vertebrate hosts by (i) aerosol, (ii) direct contact, (iii) indirect contact via fomites (objects or materials which are likely to carry infection), or (iv) arthropods acting mechanically. Three of the genera are of interest here because in nature their species are mostly transmitted mechanically by arthropods, and in a few cases by mosquitoes. Species of *Avipoxvirus* infect birds (Section 43.3.2). Species of *Capripoxvirus* infect sheep, goats or cattle, and one species has been mechanically transmitted by mosquitoes under experimental conditions (Section 43.3.4). Species of *Leporipoxvirus* infect hares, rabbits and cottontails (Leporidae) or squirrels (Sciuridae) (Section 43.3.3). The transmission of myxoma virus by mosquitoes and fleas is described in Section 43.4.

The subfamily *Entomopoxvirinae* comprises three genera, *Alphaentomopoxvirus*, *Betaentomopoxvirus* and *Gammaentomopoxvirus*; the species of all three genera infect insects. *Gammaentomopoxvirus* includes five species that infect chironomids and one species that infects culicids. When the virus *Stegomyia aegypti entomopoxvirus* (SAEV) (formerly *Aedes aegypti entomopoxvirus*) infects culicid larvae, opaque white patches are visible through the cuticle due to large occlusion bodies in the cytoplasm of infected cells (Tidona and Darai, 2002). Pox viruses listed as unassigned viruses in the family include Embu virus (ERV), isolated from mosquitoes and human blood, and Yoka poxvirus (YKV), isolated from *Stegomyia simpsoni* (Fauquet *et al.*, 2005; ICTVdb, 2010).

43.2.7 Family *Reoviridae*

The family *Reoviridae* (00.060) consists of 14 genera, species of which have been isolated from mammals, birds, reptiles, fish, invertebrates, protists, plants and fungi. Species of three genera, *Orbivirus*, *Coltivirus* and *Seadornavirus* are accepted as arboviruses or as putative arboviruses transmitted by mosquitoes, and are described later (Section 44.1.3). Species of the large genus *Cypovirus* (00.060.06) infect only insects and are not arboviruses. The name 'cypovirus' is derived from the original name of the taxon, 'cytoplasmic polyhedrosis virus', and the original acronym (CPV) is retained. A virus isolated from a continuous *Stegomyia pseudoscutellaris* cell line was assigned to a proposed new genus of *Reoviridae* by Attoui *et al.* (2005a), and the genus name *Dinovernavirus* received ICTV approval in 2006.

(a) Characteristics of cypoviruses

Genome. Comprises ten segments of linear dsRNA. Size 19–32 kbp. Genome segment reassortment occurs readily in cells co-infected with viruses of the same species. *Virions.* With icosahedral symmetry but may appear circular. The capsid, which is organized as one, two or three layers surrounding the segmented viral genome, has an overall diameter of 60–80 nm. The virions have 12 icosahedrally arranged projections, or spikes, one at each of the fivefold axes. Mature virions lack a lipoprotein envelope but may be associated with cell membranes. The entry of viruses into host cells usually results in loss of the outer layers of the capsids. The infectious forms of the virus, termed polyhedra, which appear in the cytoplasm of infected cells, are crystalline occlusion bodies containing thousands of virus particles. These µm-sized proteinaceous crystals protect the virions from hostile environmental conditions, and only dissolve at pH > 10.5 in the midgut of larval hosts, allowing the virus particles to infect the epithelial cells. *Replication.* Transcriptionally active particles (cores or double-layered particles) are released into the host-cell cytoplasm. Within these particles, asymmetric transcription of mRNA species from each dsRNA segment occurs throughout the course of infection, the products being extruded from the icosahedral apices of the particles. *Hosts.* Vertebrates, invertebrates, plants and fungi (Fauquet *et al.*, 2005; Mori and Metcalf, 2010).

Cypoviruses have been isolated only from arthropods, including 45 genera of Lepidoptera. Most cypovirus infections produce chronic disease, often without extensive larval mortality. The cypoviruses are exceptional in having transcriptionally active virions with only a single capsid shell equivalent to the 'core' particles of viruses in the other genera, particularly those with spiked cores. The single-layered capsid is composed of a central capsid shell of 57 nm diameter, which extends to 71.5 nm when the 12 spikes on the icosahedral fivefold vertices are included. The virions contain three major structural proteins and also transcriptase enzyme complexes attached to the inner surface of the capsid shell at the icosahedral fivefold vertices. Replication and virion assembly occur in the host-cell cytoplasm, accompanied by the formation of viroplasm (or virogenic stroma) which contains large amounts of viral protein. Virus particles become either singly or multiply occluded within a crystalline matrix of the virus-coded protein polyhedrin. The form of the symmetry of these polyhedra (often called occlusion or inclusion bodies) depends on both the virus strain and the host species. The polyhedrin appears to be arranged as a face-centred cubical lattice with centre-to-centre spacing of 4.1–7.4 nm. The occlusion bodies are of two types: (i) small, cuboidal bodies that contain only one or a few virions; and (ii) large, irregularly shaped bodies that contain many virions (Zhang *et al.*, 1999; Fauquet *et al.*, 2005).

Virus particles are assembled within a viroplasm in the cytoplasm of host cells, this virogenic stroma displacing the cytoplasmic organelles. With Uranotaenia sapphirina cypovirus (UsCPV), the virus particles are either randomly distributed within the viroplasm or arranged in parallel rows. The developed virions are usually icosahedral in shape,

but spherical in Culex restuans cypovirus (CrCPV), and consist of an electron-dense central core surrounded by a capsid that bears six spike-like projections of 10–15 nm length. The diameter of the viral particles varies with species. With Ochlerotatus cantator cypovirus (OcCPV), the central core measured 35 nm in diameter, while the overall particle diameter, including capsid spikes, averaged 70 nm. The virions are not enveloped. While still within the virogenic stroma, virions become occluded by the deposition around them of a protein matrix that has a crystalline lattice structure typical of polyhedrin protein. With certain cypoviruses, e.g. CrCPV and UsCPV, the protein matrix is deposited around individual particles; however, these occlusion bodies may fuse, forming larger multi-virion bodies. With OcCPV, the protein is deposited around compact groups of particles. When UsCPV infected *Ur. sapphirina*, most occlusion bodies contained a single virion, and were of 0.1 μm diameter, but a small proportion of the polyhedra were larger and contained two to eight virions. The occlusion bodies of OcCPV contained several to many virions, and were of 0.5–3.0 μm diameter. The occlusion bodies of UsCPV were described as cuboid, and those of OcCPV and CrCPV as pleomorphic (Clark and Fukuda, 1971; Andreadis, 1981, 1986; Shapiro *et al.*, 2005).

(b) Classification of cypoviruses

Initially, cypoviruses were classified into species characterized by their distinctive dsRNA electropherotype patterns (Payne and Rivers, 1976). Later, comparisons of RNA sequences confirmed the validity of that classification and 16 species were identified and named *Cypovirus 1* to *Cypovirus 16*. Of those, 15 species were isolated from lepidopterans and one from a hymenopteran. Where isolates from other host species closely resemble a named species of *Cypovirus*, they are given informal names which are treated as synonyms. Thus, the type species *Cypovirus 1* was described from isolates from *Bombyx mori*, and strains of that virus have the strain name Bombyx mori cypovirus 1 (BmCPV-1) (Fauquet *et al.*, 2005; ICTVdb, 2010).

A cypovirus isolated from wild-caught larvae of *Uranotaenia sapphirina* was named Uranotaenia sapphirina cypovirus (UsCPV) by Shapiro *et al.* (2004). Agarose gel electrophoresis of the dsRNA segments of this virus produced an electropherotype profile that differed from those of all previously recognized *Cypovirus* species, and complete nucleotide sequencing of segment ten, which encodes the polyhedrin protein, revealed no significant match with any other cypoviruses. Shapiro *et al.* (2005) treated this as a putative species of *Cypovirus*, and proposed the name Cypovirus 17, strain UsCPV-17.

A cypovirus was isolated from wild larvae of *Cx. restuans* found in Florida, and, on the basis of its electropherotype profile, and, the nucleotide and deduced amino acid sequence of its dsRNA segment ten, it was shown to be closely similar to UsCPV-17. Green *et al.* (2006) recognized it as a member of the 'Cypovirus-17 group', and designated it as species CrCPV-17. Here it is treated as a strain of Cypovirus 17. Cypoviruses isolated from four other mosquito species during the 1970s and 1980s were named Anopheles stephensi CPV, Anopheles quadrimaculatus CPV, Stegomyia sollicitans CPV and Ochlerotatus cantator CPV, but it is not known how many species they represent (Clark and Fukuda, 1971; Andreadis, 1981, 1986).

(c) Ecology and host-specificities of mosquito-infecting cypoviruses

Studies of wild mosquito populations suggested that mosquito-infecting cypoviruses have more than a single natural culicid host. In aquatic habitats in south-western Louisiana, infections of *Oc. sollicitans* and *Cx. salinarius* larvae with cypovirus were confirmed by electron microscopy (Clark and Fukuda, 1971). In a pool situated in pasture in Florida, at times when Uranotaenia sapphirina CPV was present in larvae of *Ur. sapphirina*, smaller numbers of *Ur. lowii*, *Culex erraticus* and *Anopheles crucians* larvae in the same pond were also infected with

cypovirus. When present in the *Ur. sapphirina* population at mean monthly infection rates ranging from 0.5% to 12.5%, the comparable rates in *Ur. lowii* were 1.4–2.0%, in *Cx. erraticus* 2.7–5.4%, and in *An. crucians* 0.8–3.7% (Shapiro *et al.*, 2004, 2005).

In individual habitats, the larvae of a single mosquito species may be hosts to a cypovirus alone, or to a nucleopolyhedrovirus alone, or may be dually infected with both viruses. This was the case with populations of *Oc. sollicitans* in Louisiana (Clark and Fukuda, 1971) and populations of *Ur. sapphirina* in a pool in Florida (Section 43.2.1.e).

Horizontal transmission of cypoviruses through ingestion of the occluded virus by mosquito larvae is thought to be the usual method of transmission. When uninfected first-instar larvae of *Cx. restuans* were reared in water containing polyhedra from a diseased four-instar larva, the death rate ranged from 14% to 36% (Andreadis, 1986). Vertical transmission has been demonstrated with some cypoviruses. Of 33 wild-caught adult female *Oc. sollicitans* that laid eggs, four females transmitted cypovirus vertically to the next generation, with a mean filial infection rate among the F_1 larvae of 2.8% (n = 495) (Clark and Fukuda, 1971). Of 32 *Cx. restuans* egg rafts collected from a natural aquatic habitat and allowed to hatch in the laboratory, six produced larvae infected with CPV, with the infection rate ranging from 4.8% to 15.6%. The average death rate of infected larvae and pupa was 58.2%, compared with 5.5% for uninfected juveniles from the same egg rafts (Andreadis, 1986).

(d) Laboratory studies of mosquito-infecting cypoviruses

Cypoviruses isolated from different species of mosquito are similar in structure and in the details of their morphogenesis. Usually, infections are restricted to epithelial cells of parts of the midgut, i.e. the gastric caeca and posterior midgut, but Ochlerotatus cantator CPV (OcCPV) was said to infect cells of the cardia also. Virus multiplication leads to deposition within these midgut regions of very large numbers of occlusion bodies, and collectively they produce a milky-white or blue iridescence (due to interference of light reflected from the particles) that is readily visible through the larval integument.

Laboratory observations have shown that in most mosquito hosts cypoviruses cause a largely chronic infection. Larvae of *Culex salinarius* that were heavily infected with cypovirus were usually able to pupate and emerge as apparently healthy adult mosquitoes. Even heavily infected larvae usually pupated and emerged as apparently healthy adults (Clark *et al.*, 1969). Usually, infections of *Oc. sollicitans* larvae with cypovirus were not fatal (Clark and Fukuda, 1971). Infection of *St. aegypti* larvae (an experimental host) with UsCPV did not noticeably affect the development, feeding or behaviour of the larvae. Most infected individuals pupated and emerged to the adult stage, and virus-induced mortality did not exceed 2%. Both the anterior and posterior midgut regions were infected in a proportion of the adults; the remainder had no obvious signs of infection, but bioassays showed some to be infected (Shapiro *et al.*, 2005). In contrast, *Oc. cantator* infected with CPV died during the fourth larval instar or as pupae (Andreadis, 1981).

Observations were made of the infection of healthy mosquito larvae when placed with wild-caught larvae infected with Cypovirus-17 in deionized water or in a 10 mM $MgCl_2$ solution. No larvae exposed to the virus in deionized water became infected, but when exposed in 10 mM $MgCl_2$ solution the following mean rates of infection were recorded: *Ur. sapphirina*, 13.8%; *Ur. lowii*, 9.2%; *Cx. quinquefasciatus*, 54.9%; and *St. aegypti*, 7.8% (Shapiro *et al.*, 2004). In further experiments, larvae were exposed to infection either in solutions of 10 mM $MgCl_2$ or of both 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The mean infection rate in *St. aegypti* larvae fell from 34.1% to 0.7% when $CaCl_2$ was also present (Shapiro *et al.*, 2005), and that in *Cx. quinquefasciatus* larvae fell from ~30% to ~2% (Green *et al.*, 2006). Such concentrations of Mg^{2+} and Ca^{2+} are found in some natural larval habitats (Volume 1, Chapter 6). Observations of the dual infection of wild *Ur.*

sapphirina larvae with both UsCPV and the nucleopolyhedrovirus UrsaNPV are described in Section 43.2.1.e.

(e) Characteristics of the genus Dinovernavirus

A virus first isolated from the cell line SP61, which had been established in 1974 from *Stegomyia pseudoscutellaris*, could infect that cell line in a persistent manner, causing no visible cytopathic effect. It shared most characteristics of the family Reoviridae, e.g. in virion size, length of genome and physico-chemical properties, but the genome consisted of nine distinct dsRNA segments instead of the usual ten to 12 segments. It was named Aedes pseudoscutellaris reovirus and assigned to a new genus Dinovernavirus by Attoui *et al.* (2005a). In 2006 the ITCV formally accepted the genus *Dinovernavirus*, with *Aedes pseudoscutellaris reovirus* as type species. Here the name *Stegomyia pseudoscutellaris reovirus* (SPRV) is used. Sequence comparisons showed significant amino acid identities in all proteins encoded by the genome with those of other reoviruses, particularly species of *Cypovirus* and closely related genera. Negative-staining electron microscopy showed the virions of SPRV to be single-shelled with turrets, but in contrast to the virions of cypoviruses they do not produce polyhedrin and are not occluded. SPRV did not replicate in a range of mammalian cell lines (Attoui *et al.*, 2005a).

43.3 MECHANICAL TRANSMISSION OF POX VIRUSES

43.3.1 Introduction

As noted earlier, viruses of the subfamily *Chordopoxvirinae* are transmitted between their mammalian or avian hosts by (i) aerosol, (ii) direct contact, (iii) indirect contact via fomites (objects or materials which are likely to carry infection), or (iv) mechanical (physical) actions of arthropods. Mechanical transmission is the principal mode of transmission of certain chordopox viruses which infect mammals or birds. It differs from biological transmission of arboviruses both in the manner of interaction of the virus with its arthropod and vertebrate hosts and in the types of arthropod/vertebrate relationship that the virus can exploit (Table 43.5).

Pox viruses that are transmitted mechanically by arthropods produce lesions in the skin of the host, which become densely packed with virions. If haematophagous arthropods feed through skin lesions, virions adhere to the mouthparts and can be transmitted to uninfected hosts when the arthropods feed again. Transmission can occur

Table 43.5 A comparison of the mechanical transmission of pox viruses with the biological transmission of arboviruses and the postulated mechanical transmission of arboviruses by mosquitoes. (After Fenner and Day, 1952.)

Features compared	*Mechanical transmission of pox viruses*	*Biological transmission of arboviruses*	*Mechanical transmission of arboviruses**
Source of virus	Skin lesions	Bloodstream of viraemic host	Bloodstream of viraemic host
Virus replicates in mosquito vector	No	Yes; essential for transmission	Yes, but not relevant
Extrinsic incubation period	None	Yes	Yes, but not relevant
Specificity of mosquito vectors †	None, or at most slight	Often high	Not limiting
Interrupted feeding at time of virus uptake	Increases transmission	No effect on transmission	Increases transmission
Arthropod transmission is only natural mechanism	No	Yes	Yes

*, Section 44.4.
†, In the field, host-feeding pattern determines host contact.

immediately if feeding is interrupted before satiety and then resumed on a nearby uninfected host. Otherwise it does not occur until the blood meal has been digested and the arthropod probes another susceptible vertebrate. Three genera of the subfamily *Chordopoxvirinae*, namely *Avipoxvirus*, *Leporipoxvirus* and *Capripoxvirus*, include viruses that are or may be mechanically transmitted in nature by mosquitoes. Importantly, chordopox viruses are not parasites of arthropods; they do not multiply in their arthropod vectors and are not pathogenic to them.

43.3.2 *Avipoxvirus*

Viruses of the genus *Avipoxvirus* infect birds, in which they produce two forms of lesion: (i) a more common form in which discrete, wartlike proliferative lesions develop on unfeathered areas of skin; and (ii) a diphtheritic (diphtheria-like) form in which moist, necrotic lesions develop on mucous membranes of the mouth and upper respiratory tract (van Riper *et al.*, 2002). The viruses are transmitted mechanically by arthropods, by direct contact, or through aerosols. Fowlpox virus (FWPV) has been recovered from wild *Culex pipiens* captured in the vicinity of infected chickens. In the laboratory, females of *Cx. pipiens* and *St. aegypti* that had fed through lesions on the combs of infected chickens transmitted the virus to healthy chickens when they fed again two or more days later. The virus appeared to be localized on the proboscis. Infection could be produced readily by inoculation of the proboscis 16–19 days after the mosquito became infected, but only rarely by inoculation of any other part of the body. There was no evidence of multiplication in the body of the mosquito (Kligler and Ashner, 1929, 1931).

Isolates of *Avipoxvirus* were obtained from each of four pools of *Cx. quinquefasciatus* collected in a small area of the Murray Valley in Australia. One isolate was identified as fowlpox virus; the other three were identified as closely related to fowlpox virus. A pool of *Cx. annulirostris* from the same area yielded an avipoxvirus antigenically distinct from fowlpox virus (French and Reeves, 1954).

In southern and south-eastern USA, wild turkeys (*Meleagris gallopavo*) are susceptible to infection by turkeypox virus (TKPV), which causes proliferative lesions on the skin of the head and legs and on mucous membranes. Transmission is thought to occur mechanically when mosquitoes are interrupted in blood feeding. In a Wildlife Management Area in southern Florida where wild turkeys were prone to infection with TKPV, *Culex nigripalpus* was the most abundant mosquito. Farmed turkeys that had become infected with TKPV through exposure in that area were taken to a laboratory, where *Cx. nigripalpus* took incomplete, interrupted blood meals from them before feeding again on healthy birds. All of the test birds ($n = 10$) became infected. The mosquitoes remained infective for up to four weeks (Akey *et al.*, 1981; Forrester, 1991).

Endemic Hawaiian birds are highly susceptible to one or more species of *Avipoxvirus*. In the second half of the 19th century, epizootics of 'birdpox' occurred in the lowland populations of native birds on all the major islands. Infection with unidentified species of *Avipoxvirus* proved fatal to native Hawaiian birds, and contributed to the extinction of some species. Historical evidence associated this with the introduction of *Cx. quinquefasciatus* to the Hawaiian Islands in 1826 (Warner, 1968). A 4-year investigation on the volcanic mountain Mauna Loa on Hawaii confirmed that indigenous forest birds of Hawaii were more susceptible to infection with *Avipoxvirus* than were introduced species (Table 43.6). Most lesions were on the feet, they rarely occurred on the head. Among the introduced species, only the house finch showed a high prevalence of avian pox, and that species is highly susceptible in North America also. The population density of the putative mechanical vector *Cx. quinquefasciatus* declined steeply with increasing altitude, whereas that of the indigenous birds increased. The highest prevalences of avian pox were found in the mid-altitude reaches, i.e. from about 1000 m to 1500 m, the lowest altitudes at which endemic birds were captured in any numbers. A similar association was found between altitude on Mauna Loa and prevalence of malaria in the endemic birds, and the explanation is believed to be

Table 43.6 Prevalence of infections with avipoxvirus among 3122 birds of the 11 most common species captured on the mountain Mauna Loa, Hawaii, from 1977 to 1980. (From van Riper *et al.*, 2002.)

			Birds with avipoxvirus infections		
Species *		*Number*	*Active lesions (%)*	*Inactive lesions (%)*	*Total lesions (%)*
Indigenous species					
1	Elepaio	77	9.1	10.4	19.5
2	I'iwi	107	10.3	6.5	16.8
3	Hawaii Amakihi	626	10.6	7.0	17.6
4	Omao	74	20.3	4.0	24.3
5	Apapane	601	14.1	20.8	34.9
Introduced species					
6	Spotted munia	137	0	0	0
7	Red-billed leiothrix	46	0	0	0
8	Northern cardinal	50	0	2.0	2.0
9	Japanese white-eye	1243	1.4	0.8	2.2
10	House sparrow	81	2.5	4.9	7.4
11	House finch	79	6.3	15.2	21.5

*, 1, *Chasiempis sandwichensis*; 2, *Vestiaria coccinea*; 3, *Hemignathus virens*; 4, *Myadestes obscurus*; 5, *Himatione sanguinea*; 6, *Lonchura punctulata*; 7, *Leiothrix lutea*; 8, *Cardinalis cardinalis*; 9, *Zosterops japonicus*; 10, *Passer domesticus*; 11, *Carpodacus mexicanus*.

the same. This mid-altitude region is the zone of maximum overlap of the populations of endemic bird species and of *Cx. quinquefasciatus*. The altitudinal distribution of certain endemic birds changes seasonally when they seek out nectar-bearing trees, and movement to lower altitudes during September–December explained the higher prevalence of avian pox in i'iwis during that period (van Riper, 1991; van Riper *et al.*, 2002).

43.3.3 *Leporipoxvirus*

This genus includes viruses of lagomorphs and sciurids that usually are mechanically transmitted by arthropods but that can also be transmitted by direct contact. Three species infect rabbits and hares (Leporidae) and one species infects squirrels (Sciuridae) (Table 43.7). Mosquitoes are important vectors of *Myxoma virus*, which is discussed in the next section. They are also suspected to be natural vectors of *Rabbit fibroma virus*, which infects Eastern cottontails (*Sylvilagus floridanus*) in parts of the USA and Canada. Myxoma and fibroma viruses cause localized, benign, tumour-like lesions in their natural hosts, but myxoma virus causes severe generalized disease in European rabbits. In Eastern cottontails infected with rabbit fibroma virus, fibromas appear in the skin at the sites of insect bites. These are non-malignant tumours consisting largely of fibroblasts, and infections can persist for several months. In the laboratory, species of *Anopheles*, *Culex* and *Ochlerotatus* that occurred sympatrically with *S. floridanus* acquired rabbit fibroma virus when they fed at the site of fibromas, and passed the virus to healthy *S. floridanus* on which they later fed. Experiments with *St. aegypti* showed the transfer to have the characteristics of mechanical transmission (Kilham and Dalmat, 1955; Dalmat, 1959; Fenner and Ratcliffe, 1965). Occasionally, in places where

Table 43.7 Viruses of the genus *Leporipoxvirus* (subfamily *Chordopoxvirinae*) and their natural hosts. (Data from: Fenner and Fantini, 1999; Terrell *et al.*, 2002; Fauquet *et al.*, 2005; and Wilson and Reeder, 2005.)

Virus species		*Natural hosts*		*Distribution of hosts*
Hare fibroma virus	FIBV	*Lepus europaeus*	Brown hare	Palaearctic region
Rabbit fibroma virus*	RFV	*Sylvilagus floridanus*	Eastern cottontail	Eastern and central N. America south to Venezuela
Myxoma virus	MYXV	*Sylvilagus brasiliensis*	Tapeti	Central and South America
		Sylvilagus bachmani	Brush rabbit	Western USA into Baja California Norte
		Oryctolagus cuniculus	European rabbit	Now all continents except Asia and Antarctica
Squirrel fibroma virus	SQFC	*Sciurus carolinensis*	Grey squirrel	North America

*, Shope fibroma virus.

both cottontails and mosquitoes are common, short-lasting infections with rabbit fibroma virus occur in domesticated European rabbits (*Oryctolagus cuniculus*) housed in commercial rabbitries. This virus has been used in a vaccine that was effective against infection with myxoma virus (Fenner and Fantini, 1999).

Wild Eastern grey squirrels (*Sciurus carolinensis*) have been found with extensively distributed skin fibromas (Kilham *et al.*, 1953). Most infections in wild squirrels are benign, but in an epizootic of fibromatosis in peninsular Florida the morbidity rates were high (Terrell *et al.*, 2002). In the laboratory, *Squirrel fibroma virus* was mechanically transmitted to grey squirrels by the bites of *An. quadrimaculatus* and *St. aegypti* (Kilham, 1955).

43.3.4 *Capripoxvirus*

This genus includes viruses of sheep, goats and cattle that can be mechanically transmitted by arthropods. *Lumpy skin disease virus* (LSDV) has been mechanically transmitted by mosquitoes under experimental conditions. It causes pock lesions in cattle in Africa, and is prevalent during rainy seasons when biting flies are most abundant. To test whether mosquitoes are capable of mechanically transmitting LSDV, two steers were intradermally inoculated with the virus at six sites, and females of *St. aegypti* were allowed to feed on the lesions 3 days after they had appeared. When the potentially infected mosquitoes fed again, on six susceptible cattle, 2–6 days after the infective feed, five of the cattle developed lumpy skin disease. Transmission occurred on each day from the second to the sixth after the mosquitoes had taken infected feeds. There was no evidence of virus multiplication in the mosquitoes, so transmission must have been mechanical. A later attempt to achieve mechanical transmission of LSDV between infected and susceptible cattle using *An. stephensi*, *Cx. quinquefasciatus* or species of *Culicoides* and *Stomoxys* was unsuccessful (Chihota *et al.*, 2001, 2003).

43.4 MYXOMA VIRUS AND MYXOMATOSIS

Of all inter-species systems analysed in the ecological and epidemiological literature, the association between myxoma virus, mosquitoes and rabbits is one of the most intensively studied and is one of the most informative.

In 1859, a batch of wild European rabbits arrived in Australia from England and was delivered to a property in Victoria. Some escaped and their offspring thrived well and dispersed so rapidly and widely that within a few years huge populations appeared over large parts of Australia. Myxoma virus is a virus of the western hemisphere, where it infects species of *Sylvilagus* (Leporidae). In 1950, myxoma virus 'escaped' from a trial site in New South Wales and it also

dispersed widely, infecting the wild rabbit populations and leading in time to alternating catastrophic epizootics and phases of recovery. Field investigations in Australia, led by Frank Fenner and conducted over many years into the interactions between populations of myxoma virus and its European rabbit host, came to be recognized as a classic study of the coevolution of pathogen and host, which was important even for human medicine.

43.4.1 Pathogenesis and mode of transmission of myxoma virus

(a) Pathogenesis

Following its intradermal inoculation in laboratory rabbits, myxoma virus spreads through the local lymph node to the circulation where, during a period of viraemia, virus particles are associated with leucocytes. After a period of multiplication in an internal focus, secondary dissemination occurs in the skin and elsewhere. Replication of this secondarily distributed virus causes the development of skin lesions, apparent as generalized thickenings of the face and of tissues in the anogenital region, and as scattered small papules over the body (Fenner and Woodroofe, 1953).

Infection of wild European rabbits with a highly virulent strain of myxoma virus results in a short viraemia and a generalized infection throughout the body. A lump appears at the inoculate site on the third day and later becomes large, hard and convex. Secondary skin lesions are recognizable by the sixth and seventh days; by the ninth day they are well distributed, and especially conspicuous on the snout, ears and extremities. Within these lesions, the cells of the epidermis and dermis contain many virions, typically becoming maximal on the eighth and ninth days. Thickening of the eyelids is first seen on the sixth or seventh day, the eyes are usually completely closed by the ninth day and death usually occurs on the tenth day. Under certain conditions infection can occur via the respiratory tract, but generally, in the absence of vectors, infection occurs only by entry of virus through abraded skin. Transmission between rabbits is predominantly mechanical, through virus on the mouthparts of haematophagous arthropods.

(b) Mode of transmission

Rabbits do not become infective until obvious skin lesions appear, and virus transmission follows probing through such lesions and not from engorgement with blood-containing virus. Early laboratory experiments on the transmission of myxoma virus were undertaken with species of *Sylvilagus* that are natural hosts of myxoma virus. Females of *Ochlerotatus scapularis* and *St. aegypti* that probed into skin lesions on tapeti (*Sylvilagus brasiliensis*) could transfer the virus to uninfected animals, whereas mosquitoes that probed on adjacent normal skin could not. Myxoma virus was recoverable only from the proboscis of the mosquitoes. *Stegomyia aegypti* could transmit myxoma virus up to 17 days after feeding through a skin lesion (Aragão, 1943). By means of interrupted feeding, myxoma virus was readily transmitted from infective to uninfected brush rabbits (*Sylvilagus bachmani*) by species of *Anopheles*, *Jarnellius*, *Ochlerotatus*, *Culex* and *Culiseta* (Grodhaus *et al.*, 1963).

Similar experiments were undertaken with European rabbits (*O. cuniculus*). When females of *St. aegypti* had fed through local skin lesions on viraemic European rabbits, virus could be demonstrated in the mouthparts, head and gut; in contrast, females that had fed on the ears of the same rabbits contained virions only in the gut. Only mosquitoes that had fed through skin lesions transmitted the virus to uninfected rabbits. Virus concentrations were always greater in the skin lesions than in the blood. No evidence of replication of myxoma virus was obtained over a period of 29 days in infected females of *Anopheles annulipes*, a natural vector; rather, the virus concentration decreased with time. There was no latent period between acquisition of the virus and the ability to transmit; in fact, interrupted feeding, with transfer of the mosquito from an infected to an uninfected host, usually led to transmission. When mosquitoes that had fed once through skin

lesions later probed repeatedly on the skin of healthy rabbits, their ability to induce skin lesions declined as the number of probes increased. It was estimated that some 12% of acquired infectious particles were lost in the skin at each probe (Fenner *et al.*, 1952; Day *et al.*, 1956). Measurements of transmission following multiple, successive probings by mosquitoes that previously had probed through infected skin lesions indicated that the dose of virus deposited by a mosquito was usually very small, and that infections by a single virus particle were probably fairly common. The chance of a susceptible rabbit becoming infected when a mosquito engorged was about twice that resulting from a probe (Fenner *et al.*, 1956). Titration of myxoma virus by intradermal inoculation of rabbits indicated that the average probability that a single infectious unit would produce a lesion was of the order of 0.6 (Fenner and McIntyre, 1956).

Electron-microscopic examination of sections of the mouthparts of *St. aegypti* that had fed through infected lesions showed that most of the virus particles adhered to the maxillae; the remainder were on the inner or outer surfaces of the labrum. None were observed in the food canal or salivary duct. The total number of particles, infective and non-infective, on one preparation of mouthparts was estimated to be 14,000 (Filshie, 1964).

43.4.2 Epizootiology of myxomatosis in European rabbits

Myxoma virus appears to have arisen in the western hemisphere. In Brazil, a characteristic South American strain of myxoma virus is endemic in populations of the tapeti (*Sylvilagus brasiliensis*). In these rabbits, it causes little or no viraemia but induces tumour formation in the skin, each tumour resulting from local introduction of the virus (Aragão, 1943). In California, an antigenically distinct strain of myxoma virus infects wild populations of the brush rabbit (*S. bachmani*). Two batches of wild-caught *Anopheles freeborni* contained virus resembling that in lesions on brush rabbits (Marshall and Regnery, 1960; Marshall *et al.*, 1963). Strong circumstantial evidence indicated that, in South America and California, *S. brasiliensis* and *S. bachmani* were amplifying hosts from which mosquitoes transmitted myxoma virus to colonies of domesticated European rabbits (*O. cuniculus*), causing fatal infections (Aragão, 1927; Marshall and Regnery, 1960).

Western Europe is the ancestral home of *Oryctolagus cuniculus*, but it has been widely transported by human agency. In a number of regions of the world, myxomatosis affects the population dynamics of the European rabbit, with important ecological consequences. Attempts to introduce myxomatosis into rabbit populations in Europe first succeeded in 1952, and it is now an established disease there (Fenner and Ratcliffe, 1965). Myxoma virus is readily transmitted by any insect that will bite two rabbits in succession (Aragão, 1927; Fenner and Ratcliffe, 1965). In Britain, although some mosquito species attack rabbits, feeding mainly around the snout and eyes where the virus-induced lesions occur, the principal vector of myxoma virus is the European rabbit flea, *Spilopsyllus cuniculi* (Service, 1971; Mead-Briggs, 1977). In France, culicids, ceratopogonids, simuliids and the rabbit flea all contribute to the natural transmission of myxoma virus, but the annual epizootics tend to coincide with the peak availability of mosquito vectors. In the Camargue region in southern France, myxoma virus was isolated from *Oc. caspius* and *Anopheles maculipennis* s.l. (Joubert *et al.*, 1967; Fenner and Fantini, 1999). In Australia, native mosquitoes are the principal vectors, but introduced rabbit fleas have become important vectors in some areas.

The epizootiology of myxomatosis has been investigated most intensively in Australia, where European rabbits (*O. cuniculus*) were taken from England by the first European settlers in 1788. Rabbits were taken to Australia on many occasions during the following 70 years, but all were domesticated rabbits which failed to survive as wild populations. In 1859, a batch of wild European rabbits arrived from England and was delivered to Barwon Park, a property in the Western District of

Victoria owned by Thomas Austin. Some of these escaped, and their offspring thrived so well that 8 years later, in 1867, 14,253 rabbits were shot on Austin's property. The rabbits spread across the continent at rates that varied with the density of the vegetation. They spread along the Murray–Darling river system, over an estimated distance of 125 km a year, to reach the southern Queensland border by 1866 and the Gulf of Carpentaria on the northern coast of Queensland and the Northern Territory by 1910. Within 50 years they had reached the coast of Western Australia, >2500 km from the original release site (Stodart and Parer, 1988; Thompson and King, 1994). They had long been the country's major agricultural pest.

During the years after the arrival of the 'Standard Laboratory Strain' of myxoma virus in Australia in 1937, small-scale trials were carried out in isolated localities in the semi-arid pastoral belt, where the cat flea and four species of mosquitoes were shown to be capable of acting as vectors. The released myxoma virus did not persist in the wild rabbit populations. During 1950, field trials of myxoma virus were undertaken in higher rainfall areas in southern New South Wales where, between May and November, seven liberations of the virus were made at five sites. All were disappointing until, in December 1950, myxomatosis flared up at the Balldale site, situated 7 miles north of the Murray River in New South Wales. However, an analysis of December records pointed to one or two other areas of disease which had been active before the Balldale flare-up. Almost immediately, myxomatosis was reported from other places on the Murray River system. By mid-February 1951 an epizootic of myxomatosis had arisen in narrow belts along the rivers that form the extensive Murray–Darling system and, where measured, the case fatality rates exceeded 99%. The distribution of flood-zone plants corresponded very closely with the areas in which high rabbit mortalities had occurred. The outbreaks continued until the onset of winter at the end of March 1951.

The principal vector of the 1950–51 epizootic was *Culex annulirostris*, a summer species which develops in persistent water bodies, and which had the ability to penetrate deep into rabbit burrows. During that summer season, the populations of *An. annulipes* had remained small because of the unfavourable rainfall distribution, but in the following years this species flourished and contributed to the rapid spread of the virus, possibly assisted by highly mobile vectors such as *Austrosimulium furiosum* (Simuliidae). The persistence of myxoma virus through the following winter months showed its ability to persist in rabbit populations; possibly, it was surmised, owing to ectoparasites. Within 3 years myxomatosis had spread to the eastern and western seaboards of mainland Australia, points separated by over 3500 km, and to the north it had reached southern Queensland. A survey during that period of 64,000 acres between the Murray and Murrumbidgee Rivers revealed that, while 20,000 acres were classed as heavily rabbit infested before the epizootic, only 640 acres remained so immediately after it. The dispersion of the virus was enhanced by the inoculation of many individuals in wild rabbit populations with myxoma virus of the Standard Laboratory strain or the more virulent Glenfield or Lausanne strains (Ratcliffe *et al.*, 1952; Fenner and Ratcliffe, 1965; Fenner and Ross, 1994; Hayes and Richardson, 2001).

Two different views of the natural mode of dispersion of myxoma virus through the Murray–Darling river system and beyond have been presented. Brereton (1953) plotted the progress of dispersion of the virus from the first dates on which sick rabbits were seen at locations progressively more distant from the release point. He concluded that: (i) the rate of movement was about 3 miles (4.8 km) per day; (ii) the virus dispersed steadily, not in 'jumps'; (iii) dispersion was not seasonal but continued through the winter months; and (iv) dispersion was not affected by the prevalence rate of infection. His overall conclusion was that dispersion was through the movement of rabbits, not that of vectors. Other authors concluded that dispersion of the virus reflected travel of vectors; that in some regions the virus made big 'jumps', possibly through the carriage of

vectors on wind; and that dispersion ceased in the winter (Ratcliffe *et al.*, 1952; Fenner and Fantini, 1999).

Over a period of time, myxoma virus was isolated from seven mosquito species collected in outbreak areas in Australia (Fenner and Ratcliffe, 1965), and an accumulation of evidence identified *Cx. annulirostris* and *An. annulipes* as the main vectors. (i) Frequently, those two species were locally abundant during epizootics, their numbers peaking at different times (Ratcliffe *et al.*, 1952; Myers *et al.*, 1954). (ii) The females are opportunistic in host selection, but feed readily on rabbits when present in the habitat (Lee *et al.*, 1954; Myers, 1954). (iii) In some habitats, the females rest and feed in rabbit warrens (Ratcliffe *et al.*, 1952; Lee *et al.*, 1954; Myers *et al.*, 1954). (iv) Myxoma virus was recovered from wild-caught females (Myers *et al.*, 1954; Fenner and Ratcliffe, 1965). (v) *Anopheles annulipes* transmitted myxoma virus in the laboratory (Day *et al.*, 1956; Fenner *et al.*, 1956).

In locations where epizootics of myxomatosis occurred but *Cx. annulirostris* and *An. annulipes* were absent or in low density, myxoma virus was recovered from some other rabbit-feeding species. Thus, on Mount Flora, New South Wales, where nine species of mosquitoes fed on rabbits, myxoma virus was recovered from mixed batches of *Dobrotworskyius alboannulatus* (cited as *Aedes alboannulatus*) and *Dobrotworskyius rubrithorax* (cited as *Aedes queenslandis*), the two most abundant species (Lee *et al.*, 1957). *Culex australicus* is another rabbit-biting species from which myxoma virus has been isolated in outbreak areas, and evidence suggests that it may be an important vector in southern Victoria (Fenner and Ratcliffe, 1965).

Some natural transmission of myxoma virus to rabbits could be ascribed to the stickfast flea (*Echidnophaga myrmecobii*), normally an ectoparasite of marsupials. The European rabbit flea (*Spilopsyllus cuniculi*) was first released in Australia in 1968. This allowed seasonal outbreaks of myxomatosis in mesic and semi-arid regions, but the flea did not become established in arid regions (Fenner and Fantini, 1999). A strain of myxoma virus designated Brooklands/2-93 was chosen for experimental release at sites near Cooma, New South Wales, where the European rabbit flea was the main vector, and its spread in the presence of local strains of myxoma virus was monitored by trapping and PCR analysis for 6 months. Serological evidence showed that most rabbits became infected with the introduced strain, although only relatively few were seen with myxomatosis. Use of three methods to measure the extent of spread indicated rates of spread of from 3.7 to 17.8 m per day (Merchant *et al.*, 2003).

43.4.3 Virulence and host resistance

A short review of virulence as an aspect of parasitism may be found in Section 41.5.1.

(a) Experimental protocols

Knowledge of the coevolution of myxoma virus and the European rabbit in Australia is more extensive than comparable knowledge of almost any other combination of infectious agent and wild vertebrate host. During 1951, investigations were started under the supervision of Frank Fenner, an epidemiologist, to observe the occurrence of myxomatosis and determine its epidemiology in different parts of Australia. Two variables were measured in wild populations of pathogen and host. (i) The degree of virulence of wild populations of myxoma virus. To that end, a line of genetically unselected and serologically negative rabbits, which had had no exposure to myxoma virus, was used in assays of the virulence of newly appearing strains of myxoma virus. Strains of myxoma virus were ranked into one or other of five grades according to their virulence: grade I strains were the most virulent whilst the virulence of grade V strains was highly attenuated (Table 43.8). (ii) The extent of genetically developed resistance to the virus in rabbit populations. Young rabbits were caught just after they had emerged from their burrows in early spring, before myxomatosis occurred, and when old enough they were challenged with ~5 rabbit-infectious doses of a carefully maintained line of

the Standard Laboratory Strain of myxoma virus of Grade II virulence (Fenner and Fantini, 1999).

Strains of myxoma virus were ranked into one or other of five grades of virulence. Grade I strains were the most virulent, and Grade V strains the least virulent, the virus being highly attenuated (i.e. weakened in virulence compared with the original virus) (Table 43.8). The term 'virulence grade' was defined by Fenner and Fantini (1999) as 'An artificial measure designed to obtain a measure of the lethality of myxoma virus using both proportion of a small group that were killed and their survival time as a measure of virulence'.

(b) Virulence

Fenner and Fantini (1999) defined virulence as the capacity of a virus to produce severe disease or death in a particular host species. As described in Section 43.4.2, apart from a few small-scale field trials in isolated localities in the semi-arid pastoral belt, from which myxoma virus did not persist in the rabbit populations, there were no releases of myxoma virus until 1950, when carefully managed releases of infected rabbits at five locations apparently led to little more than 'low-grade' disease in the wild populations. But, in December 1950, large numbers of sick rabbits were seen at the Balldale test site in New South Wales, and almost immediately sick rabbits were seen 15 km distant at a site on the Murray River. The highly virulent strain of myxoma virus had escaped, and the rabbit case-fatality rate was almost 100% (Table 43.9). Viral strains of reduced virulence were first isolated in 1952 and early 1953. These strains were characterized by the longer survival times of infected wild rabbits and, in laboratory tests, the survival of about 10% of genetically unselected rabbits. Strains of reduced virulence soon appeared independently in widely separated areas all over Australia. Viral strains of Grade III virulence, with inferred case-fatality rates of 70–90%, became established as the dominant group by about 1955. Then followed a long period of relative stability, during which overt myxomatosis waxed and waned in response to changes in vector activity and fluctuations in the numbers of susceptible rabbits. Despite the regular release of virus strains of Grade I virulence for the purpose of control, strains of Grades I and II were very rarely recovered from wild rabbits between the late 1950s and 1981, when systematic testing ceased. Rabbits that were infected with slightly attenuated strains were potentially infective to mosquitoes for about five times as long as rabbits infected with the fully virulent strains. The results obtained over the period 1950 to 1967 are unequivocal; myxomatosis had survived in the Australian rabbit population because natural selection had led to reduced virulence.

Strains of myxoma virus with different grades of virulence to serologically negative laboratory rabbits were found to induce different types of skin lesion in rabbits. (i) Viruses of Grade I or II virulence produced highly infective skin lesions; genetically unselected rabbits infected with Grade I virus died within 4 or 5 days of these lesions becoming infective. (ii) The epidermal cells overlying lesions induced by a highly attenuated laboratory strain (equivalent to Grade V) contained very few virus particles, and the infected animals quickly recovered; consequently the stylets of mosquitoes that probed through skin over those lesions were unlikely to become contaminated, and transmission by mosquitoes was rare. (iii) Lesions produced by the moderately attenuated Grade III strains had a high virus titre; they were infective for the remaining

Table 43.8 Criteria used for grading the virulence of strains of myxoma virus infecting populations of European rabbits in Australia. (From Fenner and Fantini, 1999.)

Mean survival time (days)	*Estimated death rate (%)*	*Virulence grade*
9–13	>99	I
14–16	95–99	II
17–28	70–95	III
29–50	50–70	IV
Not applicable	<50	V

The principal criterion used in ranking the virulence of different strains of myxoma virus was the mean survival time of groups of five or six genetically unselected and serologically negative 4–6-month-old laboratory rabbits, inoculated intradermally with about five rabbit-infectious doses of virus. Grade I strains were the most virulent; Grade V strains were the most highly attenuated (Fenner and Marshall, 1957.)

Table 43.9 The virulence of strains of myxoma virus recovered from infected, wild European rabbits in south-eastern Australia during the periods 1951–81, 1984–85 and 1992–94. Virulence grades (cf. Table 43.8) were determined on the basis of mean survival times and the estimated death rates. Grade I strains of virus were the most lethal; Grade V strains were highly attenuated. The year-by-year records of virulence grades (horizontal lines) are expressed as percentages of isolates tested. These field data are recorded over calendar periods of two or more years because in the southern hemisphere the warmer season, with higher biological activity, spans two calendar years. (From Fenner and Fantini, 1999; plus additional data for 1951–52 from Marshall and Fenner, 1960. Marshall and Fenner, 1960, also provide annual data for the years 1950 to 1958.)

	Virulence grade					
Years	*I*	*II*	*III*	*IV*	*V*	*No. of strains tested*
1950–1951	>99					1
1951–1952	33.0	50.0	17.0	0	0	6
1952–1955	13.3	20.0	53.3	13.3	0	60
1956–1958	0.7	5.3	54.6	24.1	15.5	432
1959–1963	1.8	11.1	60.5	21.8	4.7	449
1964–1966	0.6	0.6	63.4	34.0	1.3	306
1967–1969	0	0	63.0	35.7	1.6	249
1970–1974	0.6	4.6	74.1	20.7	0	174
1975–1981	1.9	3.3	67.0	27.8	0	212
1984–1985	0	17.6	76.5	5.9	0	34
1992–1994	83.3	0	0	16.7	0	48

The Standard Laboratory Strain (prototype Grade I strain) of myxoma virus, which was used to initiate the Australian epizootics, was derived from the Moses strain, which had been isolated in Rio de Janeiro in 1911, was later used by many investigators, and was taken to Australia in 1937. It had the following characteristics: mean host survival time, 10.8 days (range 8–15 days); death rate 100% (Fenner and Marshall, 1957).

lifetime of rabbits that died, or for as long as a month in rabbits that survived. Therefore, Grade III strains had great survival advantage during the winter when mosquitoes were rare. Rabbits infected with moderately attenuated strains of myxoma virus provided the most persistent source of infectivity, and such strains were the most likely to be transmitted in the field and to become dominant (Fenner *et al.*, 1956; Fenner, 1983; Fenner and Fantini, 1999).

If there had been no attenuation of the virus infecting wild populations, so that only about 1% of rabbits survived the epizootics, the disease would often have died out at the end of each summer. Further, where almost universal mortality obtained, there was no opportunity for rabbits to be selected for resistance to myxomatosis. However, less virulent strains of virus that allowed some 10% of rabbits to survive emerged quite soon, and this had two effects. (i) These less virulent strains were maintained through the winter, when mosquitoes were uncommon. (ii) Enough breeding animals survived to permit reproduction and the selection of increasingly resistant rabbits. Under the intense selection pressure the proportion of genetically resistant rabbits increased, and over the next 25 years or more there was a steady increase in host resistance such that the Standard Laboratory Strain of myxoma virus, which initially had killed over 99% of wild rabbits, was lethal for only 60%. Some of the most resistant rabbits were found in the Mallee, a low-rainfall area in the north-west of Victoria. There, although strains of Grade III virulence remained dominant, Grades I and II strains

were recovered from 8–9% of wild rabbits during the 1970s, rising to 23% by 1984–85 (Fenner and Fantini, 1999).

In 1988, the rabbit population of Australia increased enormously, suggesting to De Leo *et al.* (2002) that the 'stable equilibrium' between rabbits and myxoma virus might have been just one phase of a coevolutionary saga. About that time, a trend towards increased virulence started, and came to occur widely in temperate parts of Australia. For example, during the early 1990s, most strains of myxoma virus collected in south-eastern Australia were of Grade I virulence (Table 43.9) (Fenner and Fantini, 1999). Subsequent studies by other investigators in Australia and Europe showed that innately resistant rabbits emerged wherever myxomatosis had been present for about a decade. Further, there was a dynamic equilibrium between viral virulence and rabbit resistance, such that more virulent viruses were found in areas where the rabbit resistance was highest (Fenner and Ross, 1994).

(c) *Host resistance through immune responses*

The resistance of European rabbits to infection with myxoma virus is due to immune responses of different types. The development of an **acquired immune response** in rabbits to myxoma virus following first infection could be detected by *in vitro* tests for anti-myxoma-virus antibodies about 7 days after infection (Fenner and Woodroofe, 1953). Ordinarily, rabbits that had recovered from myxomatosis resisted reinfection for life but, occasionally, months after the initial infection, some developed localized lesions at the site of challenge inoculation. **Passive immunity** was detected from the presence of maternal antibodies in very young offspring, which protected against challenge infection (Fenner and Fantini, 1999). **Genetic resistance** was defined by Fenner and Fantini (1999) as 'resistance to the ill-effects of an infectious disease due to mutations in the genes of the host organism'. Initially, the near universal mortality provided no opportunity for rabbits to be selected for resistance to myxoma virus. But when the virulence of the dominant strains fell, so that about 10% of animals recovered, there was an opportunity for selection to favour more resistant rabbits. To test for genetic resistance in wild populations, nine or ten rabbits from a population, which were shown to be serologically negative, were inoculated with a measured dose of the Standard Laboratory Strain of myxoma virus, and the clinical effects were compared with those from similarly treated, genetically unselected laboratory rabbits. Tests carried out on rabbits from wild populations that had been exposed to myxoma virus for no or only a few generations before showed that selection for resistance to the virus occurred within a few seasons of first population exposure (Table 43.10). Rabbits with severe myxomatosis that eventually

Table 43.10 Case-fatality rates and severity of disease in groups of non-immune, wild rabbits from Lake Urana, New South Wales, after successive epizootics of myxomatosis. Challenge infection was with myxoma virus of Grade III virulence. (From Fenner and Ross, 1994; using the data of Marshall and Fenner, 1958, and Marshall and Douglas, 1961.)

No. of epizootics to which population had been exposed	*Case-fatality rate (%)*	*Clinical signs in challenged rabbits (%)*		
		Severe, incl. fatal	*Moderate*	*Mild*
0	90	93	5	2
2	88	95	5	0
3	80	93	5	2
4	50	61	26	12
5	53	75	14	11
7	30	54	16	30

recovered, or survived for over 3 weeks, would in nature have provided opportunities for mosquito transmission (Fenner and Ross, 1994; Fenner and Fantini, 1999).

(d) Coevolution of viral virulence and host resistance

The fully virulent strain of myxoma virus that initiated the outbreaks of myxomatosis in Australia was largely replaced within a few years by moderately virulent strains, which remained predominant until 1985 and possibly longer. Frank Fenner and his colleagues postulated that the long-lasting trend towards moderate virulence (Grade III) was explained by the selective advantage for transmission of strains that incapacitated but did not rapidly kill the host; rather, they induced skin lesions which persisted through the remaining life of the host, and by incapacitating the host prevented it from dislodging mosquitoes. The later demonstration of host resistance to the pathogen, resulting from selection, revealed a trade-off between virulence and resistance, the overall effects of which on populations fluctuated with time.

A mathematical model of the basic reproductive rate of pathogens developed by Anderson and May (1981) was used later (Anderson and May, 1982) to assess the relationship between the virulence of myxoma virus and its transmissibility by mosquitoes, using field data for six strains of the virus. The basic reproductive rate (R_0) was defined by:

$$R_0 = \beta N/(\alpha + b + \nu) \qquad (43.2)$$

where β was the transmission rate, N was host population density, α was the disease-induced host death rate (virulence), b was the host death rate from all other causes, and ν was the recovery rate of the infected host to the immune state. The modellers assumed that the product $\beta \times N$ was constant. Applying a functional relationship between α and ν to the expression for the basic reproductive rate, they found that R_0 was maximized by an intermediate value of α, and that the basic reproductive rate of myxoma virus was at a maximum for strains of virulence Grade IV. However, they noted that due to oversimplifications of the model its prediction of virulence Grade IV differed from the observed findings that strains of virulence Grade III had predominated (Anderson and May, 1982; May and Anderson, 1983; the findings discussed by Massad, 1987). May and Anderson (1983) appeared unaware of the resistance of the rabbit hosts to infection with myxoma virus, reported later by Fenner and Ross (1994).

From the laboratory data of Mead-Briggs and Vaughan (1975), which showed differential transmissibility by the European rabbit flea of strains of myxoma virus having different grades of virulence, Massad (1987) inferred that a curvilinear relationship exists between the transmission rate (β) and virulence (α), and by adjusting Eqn 43.2 appropriately obtained a closer fit to the field data for the period 1975–1981. The model revealed that trade-offs between fitness components prevented selection from driving transmission (β) of the pathogen to infinity, or of either its mortality (α) or recovery (ν) to zero.

Unexpectedly, four decades after the introduction of myxoma virus to Australia the situation changed. By the early 1990s, most strains of myxoma virus isolated from rabbits in temperate parts of Australia were as virulent as the original Standard Laboratory Strain. Tests carried out at that time indicated that mosquitoes that fed on resistant rabbits transmitted virus particles more effectively from lesions produced by the highly virulent strains than from lesions produced by the less virulent strains (Fenner and Fantini, 1999).

The use of myxoma virus for the biological control of wild rabbits in Australia provided a unique natural experiment on the coevolution of a virus and its host. The reappearance of highly virulent strains of myxoma virus four decades after its introduction showed that investigations of the coevolution of a pathogen and its host must be long term.

44

Arboviruses – characteristics and concepts

Arthropod-borne viruses (arboviruses) are necessarily capable of replication in both vertebrate and arthropod hosts, and are perpetuated in nature principally through biological transmission between susceptible vertebrate hosts by haematophagous arthropods. Arboviruses cause viraemia in their vertebrate hosts. They are transmitted by the bites of arthropods that become infected by feeding on viraemic hosts and that become infective after a period of extrinsic incubation during which the virus replicates. The criteria that must be proved to establish that a certain mosquito species is a biological vector of a particular arbovirus are described in Section 44.2.3.

The virus taxa that include arthropod-borne viruses are not phylogenetically related, but are treated as an unnatural assemblage called 'arboviruses' (Karabatsos, 1985; Reeves, 2001). Arboviruses are relatively simple in structure: the enveloped nucleocapsid contains (except in one species) a single- or double-stranded RNA (ssRNA or dsRNA) molecule and protein. (The exception is the tick-borne African swine fever virus (*Asfivirus, Asfarviridae*), with a genome of double-stranded DNA – dsDNA). All arboviruses have four structural proteins: (i) two named Gn or Gc (according to their relative proximity to the amino or carboxyl termini of the protein, and encoded by the M segment of the genome); (ii) a nucleocapsid protein (N) encoded by the S segment; and (iii) a large transcriptase protein (*L*) encoded by the L segment. Non-structural proteins are expressed from the S segments of certain viruses and from the M segments of others.

Arboviral infections are the cause of many diseases – in wild and domesticated animals and in humans. This chapter contains descriptions of the classification and characteristics of mosquito-borne arboviruses (family by family), descriptions of the different modes of transmission, and descriptions of the interactions of arboviruses with their mosquito and vertebrate hosts.

44.1 CHARACTERISTICS AND SYSTEMATICS BY FAMILY

Basic aspects of the genome, structure and replication of all viruses, and the fundamentals of virus classification, are described in Section 43.1. Species in five families satisfy the criteria for

mosquito-borne arboviruses, and representative arboviruses from those families are listed in Table 44.1.

44.1.1 Family *Bunyaviridae*

The family comprises five genera, of which three include arboviruses – *Orthobunyavirus*, *Phlebovirus* and *Nairovirus*.

(a) General characteristics

Genome. Segmented, comprising three separate molecules of negative- or ambisense ssRNA, designated L (large), M (medium) and S (small). Genome size: 11–19 kb. The terminal nucleotides of each genomic segment are base paired and, through hydrogen bonds, form closed, circular RNAs (and ribonucleocapsids). Segment L encodes the viral RNA-transcriptase (*L* protein); segment S encodes the nucleocapsid protein (N) and, except in nairoviruses, a non-structural protein (NSs). On segment M, a single, continuous open reading frame (ORF) encodes a precursor polyprotein, which is co-translationally cleaved to produce two mature envelope glycoproteins (Gn and Gc) and a non-structural protein (NSm). (In nairoviruses, polyprotein processing is more complex.)

Structural proteins. All bunyaviruses have four structural proteins: a nucleocapsid protein (N); a large transcriptase protein (*L*); and two envelope glycoproteins, Gn and Gc, which are named according to their relative proximities to the amino and carboxy termini of the precursor polyprotein.

Virion. Spherical or slightly pleomorphic, of 80–120 nm diameter, and enveloped with a display of surface projections, or spikes, that are embedded in a lipid-bilayered envelope (Figure 44.1). In species of *Orthobunyavirus*, the surface spikes are always of two lengths within the range 5–10 nm, embedded in an envelope 5–7 nm thick. The longer spikes are composed of the glycoprotein Gc (deduced molecular mass 108–120 kDa), and the shorter spikes of Gn (29–41 kDa); the spikes are named Gc and Gn accordingly. Formerly, these glycoproteins were named G1 (now Gc) and G2 (now Gn). In species of the genus *Phlebovirus* the surface spikes are short and of uniform length, and the glycoproteins differ little in molecular mass (Gn, 50–72 kDa; Gc, 55–75 kDa). Within the virion envelope, the three genome segments, L, M and S, are complexed with protein N to form individual L, M and S nucleocapsids. The nucleocapsids are filamentous, 200–3000 nm long and 2–2.5 nm in diameter. Their symmetry is helical. Each nucleocapsid is associated with L transcriptase protein (*L*).

(b) Classification and biology

The family *Bunyaviridae* (00.011) is one of the largest families of animal viruses, and includes almost half of the known arboviruses. It comprises five genera, two of which are not arboviruses. The species of *Tospovirus* (00.011.0.05) infect plants, can be transmitted between plants by thrips, and are capable of replicating in both plants and thrips. The species of *Hantavirus* (00.011.0.02) infect rodents, and transmission is mostly by aerosol emanations from infected rodent urine, faeces or saliva. The species of three genera are arboviruses, capable of replicating alternately in vertebrates and haematophagous arthropods. Ticks are, predominantly, the vectors of *Nairovirus* (00.011.0.03). Mosquitoes are the vectors of almost all species of *Orthobunyavirus* (00.011.0.01). Phlebotomine flies are vectors of seven species of *Phlebovirus* (00.011.01.04), and ticks the vectors of one; however, the type species of the genus, *Rift Valley fever virus*, is transmitted by mosquitoes (Table 44.2).

Species of the genus *Orthobunyavirus* are identified by the terminal nucleotide sequences of the L, M and S genome segments. The Eighth ICTV Report (Fauquet *et al.*, 2005) lists 48 accepted species of *Orthobunyavirus*, with 154 named strains, and three tentative species. The vertebrate hosts are principally rodents and other small mammals and, to a lesser extent, birds and ungulates. Mosquitoes are vectors of 42 of the accepted species, ticks are the vectors of two, and 'nest bugs' of one. Flies of the genus *Culicoides* appear to be subsidiary vectors of a number of species (Fauquet *et al.*, 2005). The viruses usually exist in silent, sylvan transmission cycles (i.e. cycles not involving human hosts), but a

Table 44.1 Examples of species of arbovirus that infect and are transmitted by mosquitoes with the addition, for two species, of strains (the names not italicized). Assigned abbreviations are included. Arboviral status according to Karabatsos (1985) or the discontinued CDC Arbovirus Catalog, except where in parentheses.

Family	*Genus*	*Viruses known from mosquitoes*	*Assigned abbreviation*	*Arboviral status*
Bunyaviridae	*Orthobunyavirus*	*Bunyamwera virus* †	BUNV	Arbovirus
		Anopheles A virus	ANAV	Probable
		Anopheles B virus	ANBV	Possible
		California encephalitis virus	CEV	Arbovirus
		Jamestown Canyon virus	JCV	Arbovirus
		Keystone virus	KEYV	Arbovirus
		La Crosse virus	LACV	Arbovirus
		San Angelo virus	SAV	Arbovirus
		Snowshoe hare virus	SSHV	Arbovirus
		Tahyna virus	TAHV	Arbovirus
	Phlebovirus	*Rift Valley fever virus* †	RVFV	Arbovirus
Flaviviridae	*Flavivirus*	*Yellow fever virus* †	YFV	Arbovirus
		Japanese encephalitis virus	JEV	Arbovirus
		Murray Valley encephalitis virus	MVEV	Arbovirus
		St. Louis encephalitis virus	SLEV	Arbovirus
		West Nile virus	WNV	Arbovirus
		Dengue virus	DENV	Arbovirus
		Dengue virus 1	DENV-1	Arbovirus
		Dengue virus 2	DENV-2	Arbovirus
		Dengue virus 3	DENV-3	Arbovirus
		Dengue virus 4	DENV-4	Arbovirus
Reoviridae	*Orbivirus*	*Umatilla virus*	UMAV	Arbovirus
		Lebombo virus	LEBV	Probable
		Orungo virus	ORUV	Probable
	Seadornavirus	*Banna virus* †	BAV	[Probable]
		Kadipiro virus	KDV	[Possible]
		Liao ning virus‡	LNV	[Possible]
Rhabdoviridae	*Vesiculovirus*	*Vesicular stomatitis Indiana virus* †	VSIV	Arbovirus
		Vesicular stomatitis Alagoas virus	VSAV	Possible
		Vesicular stomatitis New Jersey virus	VSNJV	Possible
		Cocal virus	COCV	Arbovirus
Togaviridae	*Alphavirus*	*Sindbis virus* †	SINV	Arbovirus
		Chikungunya virus	CHIKV	Arbovirus
		Eastern equine encephalitis virus	EEEV	Arbovirus
		Highlands J virus	HJV	Arbovirus
		O'nyong-nyong virus	ONNV	Arbovirus
		Ross River virus	RRV	Arbovirus
		Semliki Forest virus	SFV	Arbovirus
		Venezuelan equine encephalitis virus	VEEV	Arbovirus
		Western equine encephalitis virus	WEEV	Arbovirus

†, Type species of the genus.
‡, Tentative species.

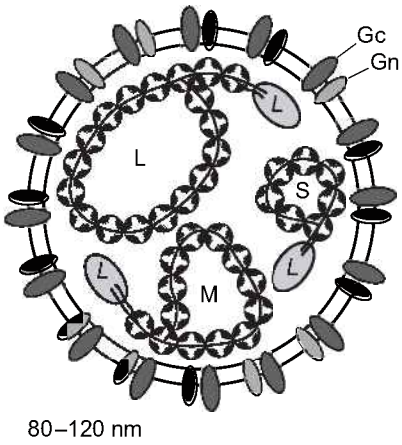

Figure 44.1 Cross-section through an orthobunyavirus (diagrammatic). The basic components of the virion are an envelope with surface projections some 5–10 nm long, and three nucleocapsids. The projections which are embedded in the bilayered envelope are glycoproteins of two types, Gc and Gn. The single-stranded RNA (ssRNA) genome is separated into three segments that differ in size and that are designated small, medium and large (S, M and L). Each of the segments is complexed with the nucleocapsid protein, forming a ribonucleocapsid that is associated with a large transcriptase protein (*L*). Thus, one ribonucleocapsid (S) contains the small-segment RNA; a second (M) contains the medium-segment RNA; and a third (L) contains the large-segment RNA. (After Schmaljohn and Hooper, 2001.)

number of them cause disease in humans or domesticated animals, notably some strains of California encephalitis virus.

The genus *Phlebovirus* is characterized by the surface structure of its virions, and by the consensus terminal nucleotide sequences of the L, M and S segments. It comprises nine species, of which seven are transmitted by phlebotomines and one by ticks. As noted above, *Rift Valley fever virus* (RVFV) (00.011.0.04.001) is transmitted by mosquitoes. There are 15 tentative species (Fauquet *et al.*, 2005).

(c) RNA segment reassortment

Viruses of different strains of a species can be present together in a host cell. If the virus is one in which the genome is divided into a number of RNA segments, sections of the genome may be exchanged in a modular fashion. Such segment reassortment has been demonstrated for viruses from most families that have genomes composed of more than one segment of RNA, including the *Bunyaviridae* (three RNA segments) and *Reoviridae* (ten to 12 RNA segments). Because genetic heterogeneity is increased by reassortment, segmentation of the genome maximizes the effects of positive selection and promotes evolutionary plasticity. Segment reassortment has only been observed *in vivo* and achieved *in vitro* for viruses belonging to the same serogroup. The ability of two viruses to reassort their RNA segments is a measure of their genetic relatedness. Within the genus *Orthobunyavirus*, reassortment has been reported between strains of *California encephalitis virus* and between strains of *Bunyamwera virus* (Pringle, 1991, 1996), and it has been described between strains of *Rift Valley fever virus* (*Phlebovirus*), as noted below.

Dual infection is a prerequisite for reassortment. In a study of superinfection, females of *Ochlerotatus triseriatus* first imbibed blood containing a temperature-sensitive mutant of La Crosse virus (LACV), and at different times afterwards they were challenged by imbibing blood-containing wild-type LACV. All females challenged at 4 h post-infection became superinfected, but the proportion superinfected steadily declined with time, to 13% at 72 h and to 0% at 7 days. Immunofluorescence staining showed that the number of midgut cells infected with LACV increased to a maximum at 96 h post-infection, and it was postulated that development progressively blocked superinfection (Sundin and Beaty, 1988). A reassortant virus derived from wild-type LACV and a temperature-sensitive mutant could be transmitted to mice by the bite of *Oc. triseriatus* (Beaty *et al.*, 1985).

To find whether reassortant viruses could be transmitted vertically, reassortants were produced by inoculating adult female *Oc. triseriatus* with temperature-sensitive mutants of both La Crosse virus and snowshoe hare virus (a strain of *California encephalitis virus*, CEV). The mosquitoes were allowed to blood feed and oviposit and their

Table 44.2 Representative mosquito-borne arboviruses, with summarized details of their vectors and vertebrate hosts. In almost all cases, diseases affecting the human hosts are zoonoses.

	Enzootic or epizootic cycles		*Transmission to dead-end hosts*	
Virus	*Established or putative vectors*	*Amplifying hosts*	*Bridge vectors*	*Dead-end hosts*
BUNYAVIRIDAE				
Orthobunyavirus				
La Crosse virus	*Oc. triseriatus*	Chipmunks, tree squirrels	*Oc. triseriatus*	Humans, deer
Phlebovirus				
Rift Valley fever virus	Species of *Aedimorphus*, *Neomelaniconion*, *Ochlerotatus*, *Culex*	Wild mammals; sheep, goats, cattle	Species of *Culex*, *Aedimorphus*, *Neomelaniconion*	Humans
FLAVIVIRIDAE				
Flavivirus				
Japanese encephalitis virus	*Culex sitiens* group species, especially *Cx. tritaeniorhynchus*	Waterbirds, pigs	*Cx. tritaeniorhynchus*	Humans, horses
Murray Valley encephalitis virus	*Cx. annulirostris*, floodwater *Ochlerotatus?* species	Waterbirds, especially Nankeen night heron	*Cx. annulirostris*	Humans
St. Louis encephalitis virus	*Cx. tarsalis*, *Cx. nigripalpus*, *Cx. pipiens*, *Cx. quinquefasciatus*	Passerine and other birds	*Cx. tarsalis*, *Cx. nigripalpus*, *Cx. pipiens*, *Cx. quinquefasciatus*	Humans
West Nile virus	*Culex* species	Birds	Aedine mosquitoes	Humans
Yellow fever virus				
E Africa – sylvatic cycle	*St. africana*	Monkeys	*St. bromeliae*	Humans
– urban cycle	*Fr. vittatus*, *Di. furcifer* group and others	Humans	–	–
W Africa – sylvatic cycle	*St. africana* group and *Di. furcifer* group spp.	Monkeys	–	–
– urban cycle	*St. aegypti formosa* *	Humans	–	–
S America – sylvatic cycle	*Haemagogus* spp., *Sa. chloropterus*	Monkeys	*Haemagogus* spp.	Humans
– urban cycle	*St. aegypti*	Humans	–	–
Dengue virus				
SE Asia – sylvatic cycle	*Do. pseudonivea*, *Do. subnivea*	Monkeys	*St. albopicta*	Humans
– urban cycle	*St. albopicta*, *St. aegypti*	Humans	–	
Africa – sylvatic cycle	*St. luteocephala*, *Di. furcifer* group	Monkeys	–	–
Elsewhere – urban cycles	*St. aegypti*	Humans	–	–
REOVIRIDAE				
Orbivirus				
Umatilla virus	Culicines	Birds	–	–

RHABDOVIRIDAE				
Vesiculovirus				
Vesicular stomatitis Indiana virus	Not known	Not known	*Oc. caspius*, *Ma. indubitans* and other biting dipterans	Cattle, horses, pigs
TOGAVIRIDAE				
Alphavirus				
Sindbis virus	Mainly spp. of *Culex* and *Culiseta*	Birds, especially passerines	? Aedine mosquitoes	Humans, orang-utans
Chikungunya virus				
Africa	*Di. furcifer*, *St. africana*	Non-human primates	*St. aegypti*	Humans
Asia	*St. aegypti*, ?*Culex* spp.	Humans	–	–
Eastern equine encephalitis virus				
North America				
Enzootic cycle	*Cs. melanura*	Birds, mostly passerines	–	–
Epizootic cycle	*Cq. perturbans*, *Oc. canadensis*, *Oc. sollicitans*, *Cx. salinarius*	Birds and small mammals	Species of *Coquillettidia*, *Aedes* and *Ochlerotatus*	Horses, humans, pigs, pheasants
Central & South America	*Culex* (*Melanoconion*), especially *Cx. taeniopus*	Small mammals and birds	*Culex* (*Melanoconion*)	Horses
Ross River virus	Species of *Ochlerotatus* and *Culex*??	Kangaroos, wallabies	Several *Ochlerotatus* spp., *Cx. annulirostris*	Humans
O'nyong-nyong virus	*An. funestus*, *An. gambiae*	Humans	–	–
Venezuelan equine encephalitis virus	Spissipes Section of *Culex* (*Melanoconion*)	Sylvan rodents // [equines]	*Ps. confinnis*, *Oc. taeniorhynchus* and species of other genera	Horses, horses
Western equine encephalitis virus (North America)	*Cx. tarsalis*, *Oc. melanimon*	Passerine birds, other birds, lagomorphs	*Ochlerotatus* spp.	Horses, humans, sciurid rodents
West Nile virus	Predominantly *Culex* spp.	Birds	*Culex* spp.	Humans, horses, birds

* Peridomestic form. ? Circumstantial evidence.
An., *Anopheles*; *Co.*, *Coquillettidia*; *Cs.*, *Culiseta*; *Cx.*, *Culex*; *Di.*, *Diceromyia*; *Do.*, *Downsiomyia*; *Hg.*, *Haemagogus*; *Ma.*, *Mansonia*; *Ne.*, *Neomelaniconion*; *Oc.*, *Ochlerotatus*; *Ps.*, *Psorophora*; *Sa.*, *Sabethes*; *St.*, *Stegomyia*.

progeny were reared. Non-temperature-sensitive, reassortant viruses were found in a small percentage of the progeny, and these could be transmitted by bite (Chandler *et al.*, 1990). To find whether mosquitoes vertically infected with one virus could become dually infected by ingesting another, *Oc. triseriatus* that were vertically infected with either temperature-sensitive or wild-type LACV were provided with blood meals containing either wild-type LACV or snowshoe hare virus. About 20% of the females became dually infected with a homologous or heterologous virus (Borucki *et al.*, 1999). The resistance to superinfection found when mosquitoes take a second infectious meal >24 h after the first must reduce the chance of superinfection occurring in nature. However, superinfection can occur when vertically infected females feed on infective hosts, so vertical infection enhances the potential for evolutionary plasticity.

Analysis of the relatedness of 23 strains of LACV by oligonucleotide fingerprinting of the tripartite RNA genomes showed them to fall into three classes – Types A, B and C (Section 45.5.1). An LACV isolate from Rochester, Minnesota, which had an S (small) RNA segment like those of type B isolates, and M (medium) and L (large) RNA segments like those of type A isolates, was considered to be a reassortant (Klimas *et al.*, 1981).

Reassortant viruses that contained heterologous S and M genomic segments were isolated from females of *Culex pipiens*; they had fed sequentially (owing to interrupted feeding) on two hamsters, one infected with an Egyptian strain and the other with a Senegalese strain of Rift Valley fever virus. The reassortant viruses replicated in the mosquitoes and were readily transmissible by bite to hamsters (Turell *et al.*, 1990). Reassortment appears to have occurred in natural populations of RVFV (Section 45.6.1.d).

44.1.2 Family *Flaviviridae*

(a) Characteristics

Genome. A single molecule of linear, positive-sense ssRNA; size, 10–11 kbp. It contains a single open reading frame, flanked by 5' and 3' terminal non-coding regions. The ORF encodes for a polyprotein of *c.* 3400 amino acids, which is cleaved co- and post-translationally. *Replication and morphogenesis*. After binding of a virion to the host-cell surface and receptor-mediated endocytosis, the nucleocapsid is released into the cytoplasm where translation and RNA replication occur. The polyprotein is cleaved by viral and cellular proteinases to produce three structural and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The N-terminal portion of the polyprotein contains the structural proteins, while the non-structural proteins are contained in the remainder. The structural proteins, which are involved in virion formation, are the capsid (C) protein, a precursor (PrM) protein from which the (M) membrane protein is cleaved, and the envelope (E) protein. The non-structural (NS) proteins provide the replicative and proteolytic functions required for virus reproduction. Transcription and genome replication occur in the host-cell cytoplasm. Viral particles are transported in cytoplasmic vesicles before release by exocytosis. *Virion*. An enveloped, spherical particle of 40–60 nm diameter, with surface projections of 5–10 nm. The RNA combines with the capsid protein to form the nucleocapsid. The envelope is a lipid bilayer containing two or three proteins. The surface glycoprotein E is responsible for viral attachment to host cell receptors, specific membrane fusion and elicitation of antibodies of different types (Fauquet *et al.*, 2005; Gould and Gritsun, 2006; reviews).

The family Flaviviridae (00.026) consists of three genera. The large genus *Flavivirus* (00.026.01), which comprises over 70 viruses, many of which are arboviruses, is antigenically and genetically distinct from the genera *Hepacivirus* and *Pestivirus*. The genus *Pestivirus* comprises species that infect pigs and ruminants and that are transmitted by direct and indirect contact. The genus *Hepacivirus* includes only *Hepatitis C virus*, which is transmitted almost exclusively by parenteral exposure to blood (Fauquet *et al.*, 2005; ICTVdb, 2010). In Section 44.1.2.b, the term flavivirus refers only to viruses of the genus *Flavivirus*.

(b) Phylogenetic relationships of flaviviruses

Investigations into the phylogenetic relationships of flaviviruses have included cladistic analyses of partial sequences of the *NS3*, *NS5* and *E* genes and other coding regions of the genomes. Analyses of partial *NS5* sequences of 71 flaviviruses showed the genus *Flavivirus* to be monophyletic. In phylogenetic trees in which *Culex fusing agent virus* (CFAV), a member of the 'insect-only' clade of flaviviruses, was used as an outgroup, it occupied a uniquely basal position (Figure 44.2) (Kuno *et al.*, 1998; Cook and Holmes, 2006). The separation of CFAV from the other flaviviruses by the longest branch in the phylogram indicated the highest degree of divergence. Hoshino *et al.* (2007) regarded members of the insect-only clade as primordial flaviviruses that emerged before other members of the genus.

Based on previously described *NS5* gene sequences, cladistic analyses were undertaken on 67 flaviviruses, of which 15 were tick borne, 39 were mosquito borne, 12 (isolated from bats or rodents) had no known vector and one was an 'insect-only' virus. In a phylogenetic tree constructed from the *NS5* gene data, as already stated, CFAV occupied a basal position, and its branch length showed its genetic distinctiveness. Disregarding CFAV, it appeared that from a hypothetical ancestor two branches had evolved – one of viruses with no known vectors (isolated from bats and rodents) and the other of vector-borne viruses (Figure 44.2). From the latter arose sister groups of tick-borne and mosquito-borne viruses (Gaunt *et al.*, 2001).

The phylogenetic groupings of flaviviruses in the *NS5* tree correlated closely but not precisely with earlier, serologically determined groupings of the flaviviruses (described in successive Reports of the ICTV). The tree displayed in Figure 44.2 shows the association of flavivirus clades with particular classes of vertebrate hosts (birds, bats, rodents, primates) and invertebrate vectors (ticks, insects), and whether they had Old World or New World distributions. The mosquito-borne flaviviruses separated into two groups: viruses that were primarily isolated from *Culex*, and viruses that were primarily isolated from aedine genera. The flaviviruses associated with *Culex* were less divergent than those associated with aedine genera, and were thought to have emerged more recently (Gaunt *et al.*, 2001). Linked to the aedine clades was a small clade of viruses isolated from bats and having no known vector, which had been designated 'No known vectors; secondary loss', implying that a capability for vector-borne transmission had been lost.

The flaviviruses associated with *Culex* are closely related genetically, and formed a single monophyletic clade, most members of which exist in mosquito–bird cycles but certain of which possibly have rodent hosts. The amplifying hosts are birds, and the viruses cause encephalitis in seriously affected tangential or dead-end hosts. Phylogenetic analyses of these viruses revealed four sub-clades with distinct geographical distributions: two in the Old World and two in the New World (Figure 44.2) (Gaunt *et al.*, 2001).

The flaviviruses that primarily were isolated from aedine genera (*Stegomyia*, *Downsiomyia*, *Dicero-myia*) formed two paraphyletic groups, one including dengue virus (DENV) and the other including yellow fever virus (YFV). The amplifying hosts are primates, and in humans, who are dead-end hosts, the viruses cause haemorrhagic disease. Flaviviruses in the aedine clade all originated in the Old World; but, of these, yellow fever virus and the dengue viruses have been transported to the New World by human agency. Once in the New World, yellow fever virus utilized species of *Haemagogus* (another aedine genus) and *Sabethes* as hosts and vectors and New World monkeys as amplifying hosts (Kuno *et al.*, 1998; Billoir *et al.*, 2000; Gaunt *et al.*, 2001).

During evolution of the genus *Flavivirus*, its species became geographically dispersed and came to occupy biologically distinct niches – characterized by their vertebrate and arthropod hosts and by the pathological effects of infection. The aedine mosquito hosts tended to feed on mammals, whereas, of the species of *Culex* that served as hosts, some fed specifically on birds but many fed on birds or

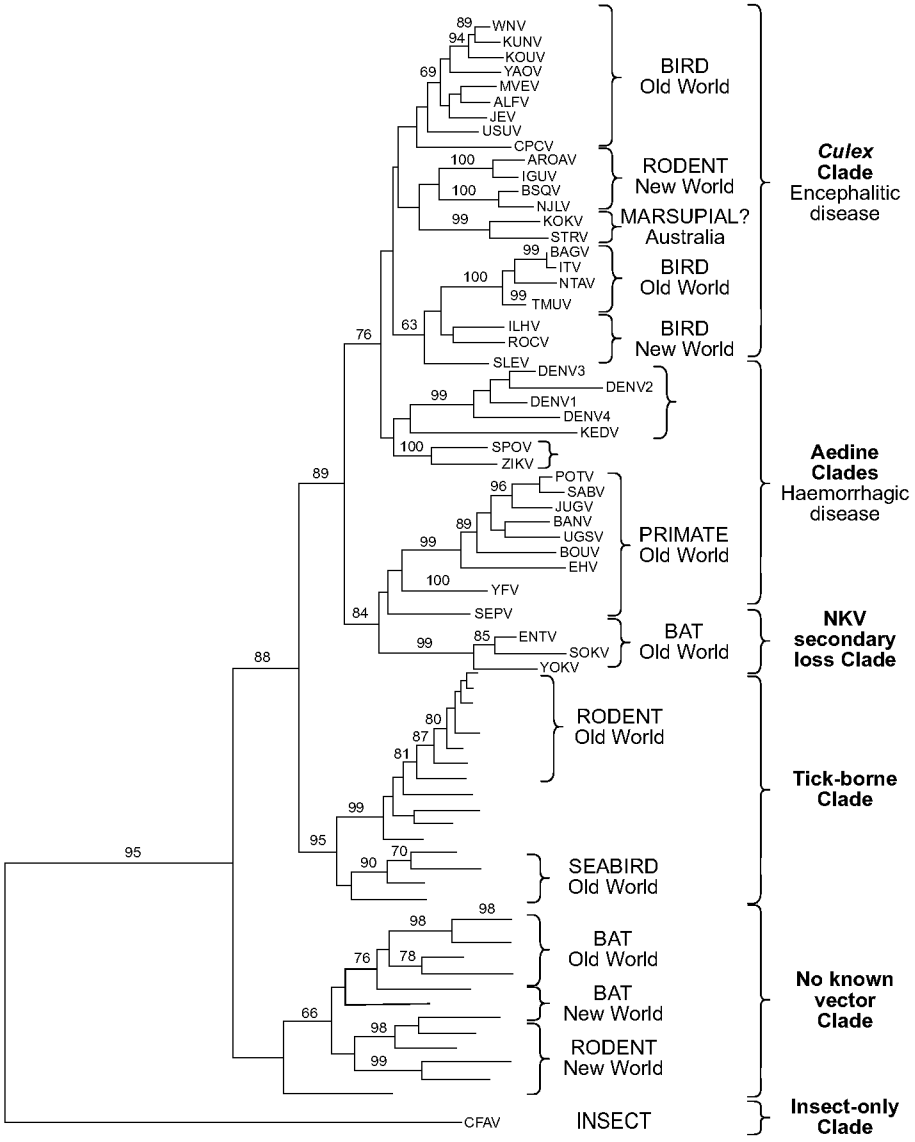

Figure 44.2 Phylogenetic tree of species (and strains) of the genus *Flavivirus*, based on their *NS5* gene sequences. *Culex fusing agent virus* (CFAV), a member of the 'insect-only' clade of flaviviruses (Section 43.2.2.a) was used as the outgroup. (After Gaunt *et al.*, 2001, but simplified.) The tree was constructed using an eight-parameter model (GTR, six parameters from the general time reversible (GTR) model of nucleotide substitution; Γ, the alpha parameter of a four-category discrete gamma distribution; and PINVAR, an invariant rate parameter). The outer series of brackets denotes the principal host clades. The inner series denotes unequivocal vertebrate-host clades, designated for monophyletic groups containing two or more virus species (or strains). To simplify the figure, the abbreviated names of the tick-borne flaviviruses are omitted. The number above each lineage shows the percentage bootstrap support for that branch. Relative branch lengths indicate percentage divergence within and between lineages. Scale bar: substitutions per site, determined from the corrected number of amino acids. The clades and sub-clades of flaviviruses as determined by cladistic analyses of the *NS5* gene sequences correspond closely with the groupings obtained serologically and in terms of vector preferences (van Regenmortel *et al.*, 2000; Fauquet *et al.*, 2005). In the explanation below, the names of viruses are italicized; the names of strains are not italicized and are placed in parentheses immediately after the names of the species to which they belong.

(Continued)

mammals according to their availability (Volume 2, Section 39.3.1). The flaviviruses transmitted by aedine mosquitoes caused haemorrhagic disease in their dead-end hosts, while those transmitted by species of *Culex* caused encephalitic disease in their dead-end hosts (Gaunt *et al.*, 2001).

The dates of divergence of parts of the phylogenetic tree could be estimated by using the rates of substitution calculated for the tick-borne viruses (4.12×10^{-5}) and mosquito-borne viruses (7.5×10^{-5} non-synonymous substitutions per site per year). This indicated that the major clades of mosquito-borne viruses arose thousands of years ago. For example, separation of the lineage that includes yellow fever virus from the lineage that gave rise to dengue virus and the Japanese encephalitis virus group occurred over 3000 years ago. However, most existing lineages diverged recently. The strains of dengue virus and of Japanese encephalitis virus arose during periods of intense cladogenesis during the last two centuries (Zanotto *et al.*, 1996).

Believing that 'attempts to resolve the higher-level taxonomic relationships of the flaviviruses through molecular phylogenetics have thus far proved inconclusive', Cook and Holmes (2006) undertook maximum likelihood analyses of amino acid sequences encoded in the *NS5* gene (73 sequences), *NS3* gene (30 sequences), and the coding region of the virus genome (23 sequences), and produced three phylogenetic trees. All analyses included the 'insect-only' viruses CFAV and KRV (Kamiti River virus) as an outgroup. Analyses were designed to elucidate the sequence of appearance in the early stages of evolution of the genus *Flavivirus* of the three main transmission groups – mosquito borne, tick borne and no known vector. Two hypotheses were consistent with these phylogenetic trees: (i) the NKV (no known vector) group diverged from a hypothetical ancestor before the arthropod-borne flaviviruses (compatible with the *NS5* gene data); and (ii) the mosquito-borne flaviviruses diverged first (compatible with the *NS3* and full genome data).

44.1.3 Family *Parvoviridae*

The family *Parvoviridae* (00.050) is separated into two subfamilies – the *Parvovirinae* (00.050.1) which infect mammals, and the *Densovirinae* (00.050.2) which infect arthropods. Densoviruses (DNVs) are grouped in the genera *Densovirus*, *Pefudensovirus*, *Iteravirus* and *Brevidensovirus*. Phylogenetic trees based on the non-structural *NS1* and *NS2* genes and the capsid *VP* gene show their distinctiveness (Zhai *et al.*, 2008; Baquerizo-Audiot *et al.*, 2009.) Their host range is limited to certain insect orders and in a very few cases to shrimps (Crustacea). They replicate in most tissues of the host, and the name reflects the accumulation of virions in dense intranuclear masses. Mosquitoes provide the known hosts of certain species of *Brevidensovirus* and *Densovirus*.

Species of ***Brevidensovirus*** have a 4 kb genome with terminal hairpins but no inverted terminal

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The clades and sub-clades determined by cladistic analysis corresponded with groups of species characterized previously by their serological characteristics. From the top of the tree downwards (but not in strict order), the serologically determined groups are as sequenced below. **Japanese encephalitis virus group**: CPCV, *Cacipacore virus*; JEV, *Japanese encephalitis virus*; KOUV, *Koutango virus*; MVEV, *Murray Valley encephalitis virus* [ALFV, Alfuy virus]; USUV, *Usutu virus*; WNV, *West Nile virus* [KUNV, Kunjin virus]; YAOV, *Yaounde virus*. **Aroa virus group**: AROAV, *Aroa virus*; BSQV, Bussuquara virus [IGUV, Iguape virus]; NJLV, Naranjal virus. **Kokobera virus group**: KOKV, *Kokobera virus*; STRV, *Stratford virus*. **Ntaya virus group**: BAGV, *Bagaza virus*; ILHV, *Ilheus virus* [ROCV, Rocio virus]; ITV, *Israel turkey meningoencephalomyelitis virus*; NTAV, *Ntaya virus*; TMUV, *Tembusu virus*. **Dengue virus group**: DENV, *Dengue virus* [DENV1–4]; KEDV, *Kedougou virus*. **Spondweni virus group**: SPOV, Spondweni virus [ZIKV, Zika virus]. **Yellow fever virus group**: BANV, *Banzi virus*; BOUV, *Bouboui virus*; EHV, *Edge Hill virus*; JUGV, *Jugra virus*; SABV, *Saboya virus* [POTV, Potiskum virus]; SEPV, *Sepik virus*; UGSV, *Uganda S virus*; YFV, *Yellow fever virus*. **Entebbe bat virus group**: ENTV, *Entebbe bat virus* [SOKV, Sokoluk virus]; YOKV, *Yokose virus*. **Outgroup**: CFAV, Cell fusion agent virus; a tentatively assigned flavivirus and member of the 'insect-only' virus group. **Unexpected placement in the tree**: SLEV, *St. Louis encephalitis virus*, which is well substantiated as a member of the Japanese encephalitis virus serogroup, is placed in this tree as a sister virus to the Ntaya virus group. Later cladistic studies (e.g. Baleotti *et al.*, 2003) place SLEV in the JEV serogroup. NKV, No known vector (secondary loss clade).

repeats. Populations of virions encapsidate positive and negative strands, but 85% of strands are of negative polarity. Two viruses with mosquito hosts have received formal recognition as species of *Brevidensovirus*. Both formerly included *Aedes* in their names, but here they are named *Stegomyia aegypti densovirus* (SaeDNV) and *Stegomyia albopicta densovirus* (SalDNV). A third species, Stegomyia pseudoscutellaris densovirus (SpDNV), was treated as a 'tentative species in the genus' (Fauquet *et al.*, 2005). Three different neighbour-joining phylogenetic tree-involving species of all four densovirus genera showed Stegomyia albopicta DNV appearing in three separate lineages (SalDNV-1, SalDNV-2 and SalDNV-3) in each tree. From their affinities, the three lineages were thought to represent two distinct species of *Brevidensovirus*. Two putative densoviruses were included in the same cladistic analyses. One had been isolated from adult *Culex pipiens pallens* in China, and named Culex pipiens pallens DNV (CppDNV). In each of three trees, CppDNV separated with SaeDNV, and was thought to represent a novel variant of that species within the genus *Brevidensovirus*. Haemagogus equinus DNV, derived from a cell line of *Haemagogus equinus*, separated in two of the three trees with two of the SalDNV lineages (Zhai *et al.*, 2008).

Species of ***Densovirus*** have a genome of c. 6 kb, with long terminal repeats and ambisense organization, and populations of the virions encapsidate equal numbers of positive and negative strands. The two formally accepted species have lepidopteran hosts (Fauquet *et al.*, 2005). A virus isolated from diseased larvae in a laboratory strain of *Culex pipiens*, originally collected in France and so the subspecies *Cx. pipiens pipiens*, had the main molecular and biophysical characteristics of a densovirus, and was named Culex pipiens DNV (CpDNV). The lack of sequence homology between CpDNV and AalDNV (*Brevidensovirus*), and the lack of antigenic cross-reactivity between their capsid polypeptides, showed them to be phylogenetically distinct. In contrast, CpDNV shared a slight but distinct antigenic relationships with *Junonia coenia densovirus* (Lepidoptera), the type species of *Densovirus* (Jousset *et al.*, 2000).

From cladistic analyses of viruses from all four genera of Densovirinae, two neighbour-joining phylogenetic trees were produced based on NS1 and VP1 proteins. Also included in the analyses was CpDNV isolated from the same laboratory strain of *Culex pipiens* as above. In both trees, CpDNV was situated distant from the *Brevidensovirus* clone but as a sister group to *Densovirus*. The CpDNV genome shared the main characteristics of viruses belonging to the genus *Densovirus* and with lepidopteran hosts: an ambisense organization, the presence of inverted terminal repeats (ITRs), and significant identities at both capsid and *NS* gene levels. However, CpDNV deviated from that model in at least five genomic domains: (i) the terminal hairpin of the ITR; (ii) the capsid VP proteins; (iii) the ORFs of the *NS1* and *NS2* coding sequences; (iv) the number of promoters controlling the expression of *NS* genes; and (v) the location of the TATA boxes. Baquerizo-Audiot *et al.* (2009) concluded that, taken together, these structural differences strongly suggest that CpDNV differs sufficiently from other densoviruses in both its replication and expression strategies to be considered as the prototype of a possible new genus within the subfamily *Densovirinae*.

44.1.4 Family *Reoviridae*

The characteristics of the family *Reoviridae* (00.060) were summarized earlier (Section 43.2.7). The family comprises 12 genera, which infect animals, plants, fungi or protists. Species of the genus *Cypovirus* (00.060.0.06) infect only arthropods and are not arboviruses. Species of the genera *Orbivirus* (00.060.0.02), *Coltivirus* (00.060.0.04) and *Seadornavirus* (00.060.0.10) have biological characteristics of arboviruses, but for some species the evidence for arboviral status is incomplete. The virions of these three genera are similar in terms of particle diameter, icosahedral symmetry, possession of short projections and absence of a lipid envelope (although a covering may be acquired from host membrane). The genome of *Orbivirus* consists of ten segments of dsRNA, the genomes of *Coltivirus* and *Seadornavirus* of 12 segments of dsRNA. The two species of *Coltivirus*

are transmitted by ticks, and will not be considered further.

The 21 species of *Orbivirus* replicate in and are transmitted by mosquitoes, ceratopogonids, phlebotomines or ticks. Full arboviral status has been accorded to three *Orbivirus* species: *Bluetongue virus* (the type species, which infects ruminants) and *African horsesickness virus*, both transmitted by ceratopogonids; and *Umatilla virus*, which infects birds and is transmitted by culicine mosquitoes. The other species are ranked as 'probable' or 'possible' arboviruses (Table 44.1). Among these, *Lebombo virus* (probable arbovirus) is associated with rodents, humans and culicine mosquitoes, while *Warrego virus* (possible arbovirus) is associated with marsupials, anopheline and culicine mosquitoes and *Culicoides*. *Orungo virus* (probable arbovirus) is associated with artiodactyls, primates and culicine mosquitoes (Fauquet *et al.*, 2005). In Central and West Africa, *Orungo virus* has been isolated from species of *Culex*, *Diceromyia*, *Stegomyia* and *Anopheles*. It was isolated from males of species of *Diceromyia* that had been attracted to monkey bait in the forest canopy in Côte d'Ivoire, indicating vertical transmission (Cordellier *et al.*, 1982). In arthropods, Orbivirus infections have no evident harmful effect; in vertebrates the effects of infection range from inapparent to fatal.

Certain viruses isolated from pools of *Culex* or *Anopheles* species during a surveillance programme conducted in Indonesia in 1980–1981 were of uncertain affinity. They were 12-segmented dsRNA viruses; most had an electropherotype profile of two groups of six segments (6-6), while one, from a pool of *Culex fuscocephala*, had a unique 6-5-1 profile (Brown *et al.*, 1993). Complete sequence determination and genetic analysis showed them to be reoviruses of distinct genotypes and led to the erection of a new genus, *Seadornavirus* (Attoui *et al.*, 1998, 2000). *Seadornavirus* now includes three species: the type species *Banna virus* (electropherotype profile 6-6), which was transferred from *Coltivirus*; *Kadipiro virus* (profile 6-5-1); and *Liao ning virus* (profile 6-6) (Attoui *et al.*, 2005b, 2006).

Banna virus was first isolated from serum and cerebrospinal fluid of encephalitis patients in Banna Prefecture, Yunnan Province, southern China. Persons infected with Banna virus had flu-like symptoms, myalgia, arthralgia and encephalitis (Xu *et al.*, 1990). It has been isolated from species of *Culex* and *Anopheles* in Indonesia, and from species of *Culex* in Vietnam (Brown *et al.*, 1993; Nabeshima *et al.*, 2008).

Kadipiro virus was isolated from *Culex fuscocephala* in Indonesia; no vertebrate host is known (Brown *et al.*, 1993). Liao ning virus was isolated from *Ochlerotatus dorsalis* in the Liaoning Province in north-eastern China. It could replicate in both mosquito and vertebrate cell lines (Attoui *et al.*, 2005b).

44.1.5 Family *Rhabdoviridae*

(a) Characteristics and classification

Genome. A single molecule of linear, negative-sense ssRNA. Size, 11–15 kb. *Virion*. Viruses that infect vertebrates are bullet shaped or cone shaped; those that infect plants appear bacilliform when fixed. Length 100–430 nm, diameter 45–100 nm. Outer surface covered with projections, 5–10 nm long and 3 nm in diameter, formed of the viral glycoprotein (G). The nucleocapsid, which exhibits helical symmetry, consists of an RNA and N-protein complex associated with two polymerases, proteins (L) and (P). A lipid envelope contains the glycoprotein (G). *Replication*. Transcription and genomic replication occur in the cytoplasm, virion assembly in the plasma membrane. The 42S RNA molecule is transcribed into five mRNAs coding for five polypeptides designated L, G, N, P and M. The single, linear molecule of RNA is complexed with about 1200 molecules of nucleocapsid protein (N) that are arranged like beads on a string, and the complex is coiled into a tightly packed helix. The peripheral-matrix protein (M), which surrounds the nucleocapsid core, is thought to regulate genomic RNA transcription. *Hosts*. Vertebrates and plants, according to genus. Plant rhabdoviruses usually penetrate cells through points of damage caused by feeding insects, but certain of them replicate also in their

insect vectors (Rose and Whitt, 2001; Fauquet *et al.*, 2005).

The family *Rhabdoviridae* (01.062), of the order *Mononegavirales*, comprises six genera, of which the genus *Vesiculovirus* (01.062.0.01) is of interest here. It consists of nine species, and there are 19 tentative species. Most, and possibly all, accepted vesiculoviruses cause vesicular stomatitis in livestock. *Sigma virus* (SIGMAV) is a rhabdovirus that is unassigned to genus. Not an arbovirus, it infects wild populations of *Drosophila* in which it is transmitted vertically, and provides the best understood example of stable and non-stable infections (Section 41.3).

(b) Biology of vesiculoviruses

Vesiculoviruses have been recovered from mammals, fish and insects. There are nine accepted species of *Vesiculovirus*, of which several are associated with vesicular disease in livestock. The best known species are four related New World viruses which infect cattle and horses, and in some cases other domesticated animals. They are the type species *Vesicular stomatitis Indiana virus* (VSIV), *Vesicular stomatitis New Jersey virus* (VSNJV), *Vesicular stomatitis Alagoas virus* (VSAV), and *Cocal virus* (COCV). VSNJV and VSIV are enzootic in Central America and northern South America, but they can disperse and epizootics have occurred from Argentina to Canada. VSAV has been associated with vesicular disease in horses and mules in Brazil, and COCV with the same in Argentina.

The disease vesicular stomatitis takes its name from the vesicles (blisters) that often form during the acute phase of infection at a variety of sites on the body, including the gums, lips and teats. Its symptoms resemble those of foot-and-mouth disease. The lesions noted in cattle infected with VSVNJ include exfoliation of mucous membranes, swelling of the nostrils and muzzle, sloughing of muzzle skin, nasal discharge and vesicular lesions on the coronary band (the soft region at the top of the hoof). Infection can cause debilitating lameness in horses and pigs, and loss of milk production in cows. Pigs develop a fever 1–3 days after infection, and vesicles of up to 3 cm diameter appear on the snout and interdigital areas, but the animals normally recover within 2 weeks (Letchworth *et al.*, 1999; Comer, 2001; reviews).

Enzootics of vesicular stomatitis occur in lowland tropical areas in Central and South America as a result of infections with Vesicular stomatitis Indiana virus (VSIV) and Vesicular stomatitis New Jersey virus (VSNJV). In more temperate areas these two viruses cause epizootics, and large-scale epizootics have been reported from Argentina to Canada. VSIV and VSNJV have been isolated from a wide range of non-domesticated animal species, including deer, lynx, bear, deer mice and bats. Exposure of humans to infected animals can lead to an acute, self-limiting influenza-like illness, usually without vesiculation (Letchworth *et al.*, 1999, review; Comer, 2001; Rose and Whitt, 2001).

(c) Transmission

Vesicular stomatitis Indiana virus and Cocal virus are classed as arboviruses (CDC Arbovirus Catalog), but viraemia has not been described in any domesticated animals after natural or experimental infection (Mead *et al.*, 2004b). Certain of the vesiculoviruses that infect livestock have been isolated from naturally infected arthropods, notably blackflies, phlebotomine sandflies and mosquitoes. The relatively few reports of isolations of vesiculoviruses from wild-caught mosquitoes include VSIV from a pool of *Ochlerotatus* species (probably *Oc. dorsalis*) (Sudia *et al.*, 1967); VSNJV from a pool of *Mansonia indubitans* (Calisher *et al.*, 1983); and Malpais Spring virus, a tentative species of *Vesiculovirus*, from single pools of *Ochlerotatus campestris* and *Psorophora signipennis* (Clark *et al.*, 1988).

During an epizootic of vesicular stomatitis in cattle and horses in Colorado, insects were collected by light trap or sweep net and screened for virus. VSNJV was recovered from houseflies and eye gnats (*Hippeletes* spp., Chloropidae), which are known to feed around the eyes and mouth lesions (Francy *et al.*, 1988).

Female *Stegomyia aegypti* that imbibed sugar solution containing VSIV ingested an estimated

3 µl containing about 7×10^4 PFU during 3 days. Extracts from homogenated mosquitoes showed that at 2 days post-infection the virus content had fallen slightly to 3.2×10^4 PFU, but it later increased, the titres on day 7 being 8.7×10^5 PFU, and on day 15 being 4.6×10^5 PFU. On day 21 the titre was still high at 1.8×10^4 PFU. On four occasions, infected females fed on suckling mice. On day 4 post-infection, the mice were unaffected, but on days 5, 7 and 15 post-infection each bitten mouse died, and VSIV could be extracted from their brains (Mussgay and Suárez, 1962).

An attempt to determine the vector capacity of *St. aegypti* for VSIV was made in the laboratory. 'Baby' mice were given intraperitoneal inoculations of 2000 PFU, and at 24, 36 and 48 h post-inoculation different batches of 16 female mosquitoes (totalling 48) were allowed to feed on the inoculated mice. Then, on days 3, 6, 10 and 15 after the inoculations, each of the batches of mosquitoes was allowed to feed on 16 healthy mice, and the numbers of mice infected were recorded. Thus, each batch of 16 mice had four opportunities for transmission to healthy mice (total per batch 64, grand total 192). After the day-15 blood meals all 48 mosquitoes were triturated for virus titration. The results are difficult to interpret because virus was found in the blood of only 16 of the 48 mice, and recovered from only 15 of the 48 mosquitoes. Summarizing the results, on days 3. 6, 10 and 15 post-infective blood meal, totals of 3, 6, 8 and 5 mosquitoes, respectively, transmitted VSIV to uninfected mice, a grand total of 22 out of 192 contacts (Bergold *et al.*, 1968).

Published reports of infection of mosquitoes by intrathoracic inoculation of pathogens are largely ignored in this volume, because the outcomes may differ markedly from those that follow natural transmission. However, reported findings from parenteral infection of sandflies with VSIV and of blackflies with VSNJV are of some interest and are described here.

In Panama, VSIV was isolated from wild-caught phlebotomine sandflies of the genus *Lutzomyia*, and was isolated also from spider monkeys and climbing rats placed as sentinel animals in forest (Tesh *et al.*, 1974). Following intrathoracic inoculation of sandflies, VSIV replicated in *Lutzomyia trapidoi* and *Lutzomyia ylephilator* but not in two other *Lutzomyia* species. VSIV could be isolated from all life-cycle stages and both sexes of *L. trapidoi* and *L. ylephilator*, indicating vertical transmission, with the titre increasing with developmental stage. The maternal infection rates in the two species ranged from 20% to 27%. Infected F_1 females of *L. trapidoi* transmitted VSIV to 'susceptible animals' by bite, and transmitted the virus transovarially to 34% of their offspring, the F_2 generation. Tesh and Chaniotis (1975) concluded that with such low maternal infection rates VSIV could not be maintained by vertical transmission alone.

Females of the blackfly *Simulium vittatum* that had been intrathoracically inoculated with VSNJV were placed 3–4 days later on either the snout or abdomen of pigs and allowed to blood feed. VSNJV-specific neutralizing antibody appeared in the pigs bitten on the snout, and those pigs developed vesicular lesions in that region, but no virus could be isolated from their blood. No positive responses were shown by pigs bitten on the abdomen. To test whether blackflies could become infected with VSNJV by feeding on vesicular lesions, non-infected male and female flies were caged over the snouts of infected pigs. The virus was isolated from 16–18% of females and 70% of males. The VSNJV titres increased markedly in females kept for 5 days post-infection, but not in the males. When uninfected male or female flies fed at the same time as infected females on uninfected pigs, the virus was later found in the initially uninfected flies, but only briefly in the males (Mead *et al.*, 2004a,b).

On cattle infected with VSNJV, lesions are usually observed in the mouth, nostrils and coronary bands. When female *S. vittatum* parenterally infected with VSNJV fed at such sites on uninfected cattle, infection occurred that was characterized by strong, local replication and the formation of vesicular lesions, and induced production of high titres of neutralizing antibody.

In contrast, when infected flies fed on the flank or neck, local replication was poor, lesions were not observed and neutralizing-antibody titres were low (Mead *et al.*, 2009).

From their observations on the transmission of VSNJV between blackflies and pigs, Mead *et al.* (2004b) concluded that haematophagous insects can become infected with vesiculovirus by feeding from vesicular lesions on infected hosts. That contrasts with the usually accepted understanding that for horizontal transmission vectors must first feed on viraemic hosts. They also concluded that non-haematophagous insects such as houseflies and eye gnats that feed on the eyes and mouth lesions might acquire VSNJV by feeding simultaneously with infected blackflies.

44.1.6 Family *Togaviridae*

Genome. A molecule of linear, positive-sense ssRNA, capped at the 5'-terminus and polyadenylated at the 3'-terminus. Size, 10–12 kb. *Virion.* Spherical; diameter about 70 nm, with a lipid envelope containing heterodimeric glycoprotein spikes, 6–10 nm long, composed of two glycoproteins. The envelope tightly encloses an icosahedral nucleocapsid, of 40 nm diameter. The nucleocapsid is composed of 240 copies of the capsid protein, organized in icosahedral symmetry, and the genomic RNA. *Replication.* Genetic transcription and replication take place within the cytoplasm of host cells, and replication is completed when virions bud through the plasma membrane. The genomic RNA serves as the mRNA for the non-structural proteins of the virus, which replicate viral RNA and probably act in association with cellular proteins. *Hosts.* Vertebrates and invertebrates (Fauquet *et al.*, 2005).

The family *Togaviridae* (00.073) consists of two genera: *Rubivirus* (00.073.0.02) with a single species (*Rubella virus*), and *Alphavirus* (00.073.0.01) with 29 species. A phylogenetic tree generated from partial E1 envelope glycoprotein gene sequences of all alphavirus species except one grouped the alphaviruses into four clusters. One cluster consisted only of Salmon pancreas disease virus. A second cluster included (among other viruses) Barmah Forest, chikungunya, O'nyong-nyong, Ross River and Semliki Forest viruses; a third included Highlands J, Sindbis, Whataroa and Western equine encephalitis viruses; and the fourth included Eastern equine and Venezuelan equine encephalitis viruses (Fauquet *et al.*, 2005).

As a group, alphaviruses are found on all continents except Antarctica and on many islands, but most individual viruses have a limited distribution. Exceptionally, Sindbis virus has been isolated from its hosts in many regions of Europe, Africa, Asia, the Philippines and Australia, while Western equine encephalitis virus occurs discontinuously from Argentina to Canada. Alphaviruses have a wide vertebrate host range; some have species of birds and others have species of mammals as their principal vertebrate amplifying host, but most are able to replicate and so multiply in mammals. Most alphaviruses are able to replicate in and be transmitted by mosquitoes, which function as vectors. Characteristically, they are transmitted by culicine mosquitoes; O'nyong-nyong virus is exceptional in being transmitted by anophelines (Section 44.8). Alphaviruses produce a lifelong infection in mosquitoes, but an acute and usually short-duration infection in vertebrates. A number of alphaviruses can infect humans as dead-end hosts. In the Old World, such infections cause fever and a painful arthralgia but are not life threatening. In the New World, infections with Eastern, Western and Venezuelan equine encephalitis viruses in humans and horses can prove fatal in a percentage of cases (Williams *et al.*, 1965; Strauss and Strauss, 1994; Schlesinger and Schlesinger, 2001; Woodall, 2001; Fauquet *et al.*, 2005).

44.2 ARBOVIRAL TRANSMISSION CYCLES

Arboviruses persist in nature by alternately infecting vertebrate and arthropod hosts, being adapted to two very different host types. Passage between the arthropod and vertebrate hosts is by horizontal transmission (Section 41.2.2). In its simplest form, an arboviral cycle involves two episodes of transmission and two phases of replication: (i) the

vectors (most often mosquitoes or ticks) ingest blood from viraemic vertebrates, and virions invade their tissues; (ii) the virus replicates within the arthropod hosts, and virions accumulate in the salivary glands; (iii) when infected arthropods feed again, virions are passed in saliva to the vertebrate hosts, infecting them; and (iv) the virus replicates in the vertebrate host, accumulating within the bloodstream and internal organs.

The fact that vertebrates that become infected by an arbovirus and recover are immune for life, or at least for an extensive period, and so must be succeeded as hosts by susceptible individuals, restricts the means by which arboviruses may be perpetuated through time. As one example, in wild-caught house sparrows that were infected with WNV (West Nile virus) by needle inoculation, neutralizing antibodies provided immunity against reinfection for up to 36 months (Nemeth *et al.*, 2009a). In contrast, mosquitoes, once infected, remain infective for life (Section 44.8.1.g).

44.2.1 Introduction

Most of the arboviruses listed in Table 44.2 cause zoonoses, i.e. pathogen-induced diseases of wild or domesticated animals that can be spread to humans. Usually these viruses are perpetuated through enzootic cycles, being transmitted between amplifying hosts by susceptible mosquito species that regularly feed on them. In some instances, the viruses may be ingested by bridge vectors, i.e. mosquito species that include humans or domesticated animals in their host range in addition to their wild vertebrate hosts. For most such arboviruses, humans and domesticated animals are dead-end hosts that do not produce viraemias of sufficient titre and duration to permit further transmission. A small number of arboviruses, including dengue virus and yellow fever virus, exist not only in enzootic cycles in the environments of their natural amplifying hosts and vectors, but also in large urban areas where humans are the sole host species and the vectors are synanthropic.

Where populations of a virus and populations of its vertebrate host coexist and interact, selective forces may induce genetic changes in one or both populations that cause significant changes to the phenotype. For example, selective forces acting on populations of myxoma virus infecting populations of the European rabbit in Australia produced, from time to time, changes to the viral genotype that were apparent as changes of serotype, and that sometimes also affected the virulence of the virus (Section 43.4.3). Clearly, results obtained from laboratory experiments that utilize long-colonized strains of a vertebrate host and of a mosquito vector, and cultured arbovirus, are of little or no value in attempts to model the natural situation.

44.2.2 Types of vertebrate host

Not all vertebrate species that are susceptible to infection by an arbovirus are involved in its transmission cycle, and terms have been coined to distinguish the different relationships between vertebrates and the arboviruses that infect them. As noted earlier in Section 41.1.2, 'competent hosts' are vertebrate species that develop a viraemia or parasitaemia that is of sufficient titre and duration to infect blood-feeding vectors, whereas 'incompetent hosts' are species that, when infected, develop a viraemia or parasitaemia that is of insufficient titre and duration for vectors feeding on them to become infected. In reports of investigations into the transmission of arboviruses by mosquitoes, the term 'amplifying host' (or 'amplification host') is usually used in place of 'competent host', and the terms 'dead-end host' or 'tangential host' are used in preference to 'incompetent host'.

The term **amplifying host** describes bird or mammal species which, when infected by a particular arbovirus, develop a viraemia of sufficient titre and duration to infect blood-feeding females of a vector species. The term is commonly applied to the members of an amplifying host species. As a consequence of mosquitoes feeding on amplifying hosts, the proportion of infected mosquitoes in the vector population becomes amplified, as subsequently do infection rates in populations of susceptible host species, allowing completion of the

transmission cycle (cf. Figure 45.33). Amplifying hosts are described as reproducing annually or more frequently and providing large numbers of new susceptibles concurrent with vector activity. Susceptible amplifying hosts may not be present year round (Higgs and Beaty, 2005).

The term **reservoir host** refers to vertebrate species, or to individuals of such species, that survive for a long period after becoming infected by a particular infectious agent, while continuing to be a source for transmission of that agent. It is appropriate for the vertebrate hosts of certain eukaryotic parasites, e.g. filarial nematodes, but its use for the vertebrate hosts of arboviruses cannot be justified. The term is rarely defined by those who use it, but a review article by Higgs and Beaty (2005) lists the characteristics of vertebrate species that are regarded as reservoir hosts of arboviruses. In fact, certain of those characteristics negate that possibility. For example, reservoir hosts should: (i) experience no untoward effect from infection; (ii) develop a high-titred viraemia of long duration, which can lead to the infection of many vectors; and (iii) be involved in the maintenance of the virus over long periods of time. In reality, the vertebrate hosts that develop high-titred viraemias often suffer pathogenic effects and, critically important, individuals that survive infection become immune for life and develop no further viraemias.

The characteristics of the so-called reservoir hosts of particular arboviruses cannot be distinguished from those of amplifying hosts, therefore, the term 'reservoir host' is redundant. However, an arbovirus may have more than one species of amplifying host, and it is necessary that the species that are more important and less important for perpetuation of the virus should be distinguished from one another. Two parasitological terms that might be used to distinguish them are 'primary host' and 'secondary host'.

The ability of bird species to serve as supposedly 'reservoir hosts' of West Nile virus was measured in laboratory experiments in which birds were brought into close contact with two batches of mosquitoes, first allowing transmission of an arbovirus to the birds by infective mosquitoes, and later allowing transmission from the birds to healthy mosquitoes (Komar *et al.*, 2003). The outcome of an experiment was a quantitative measurement named the 'index of reservoir competence'. That measurement was appropriate to amplifying hosts and is renamed here. The **index of amplifying competence** (C_i) is the product of three factors, such that $C_i = s \cdot i \cdot d$, where s (susceptibility) is the proportion of test birds that become infected, i (mean daily infectiousness) is the proportion of exposed vectors that become infective per day, and d (duration of infectiousness) is the number of days that a host maintains an infectious viraemia of sufficient titre. In nature, specific host–pathogen relationships are a product of coevolution; therefore, experiments designed to provide realistic indices of amplifying competence should correspond to the natural situation to the extent that both the mosquito vectors and the vertebrate hosts are wild caught from a locality in which the test strain of the arbovirus is endemic. (See also Section 45.7.7.b).

Rejection of the concept of reservoir hosts of arboviruses removes what has been an important element in models of the modes of perpetuation of some arboviruses, especially of their perpetuation through long periods of adverse climatic conditions. In such cases, further field investigations may be necessary to develop more realistic models.

A **dead-end host** or **tangential host** is a bird or a mammal species that, when infected with a particular arbovirus, sustains a viraemia that is of insufficient titre and duration to permit transmission to blood-feeding mosquitoes (cf. Figure 45.33). The presence of a population of dead-end hosts in an area can reduce the rate of infection of a vector population, and so also the rates of infection among the amplifying hosts.

44.2.3 Vector competence

The different vectors of an arbovirus are likely to differ in **vector competence**, i.e. a combination of: potential to acquire an infection from a viraemic host; potential to permit rapid replication and dissemination of the virus with accumulation of

virions in their salivary glands; and ability to transmit the virus and infect a new host. A quantitative measurement of vector competence is the proportion of vectors that, having fed on an infective vertebrate host, can subsequently transmit the pathogen to another host individual (Chamberlain *et al.*, 1954; Burke and Leake, 1988; Vaidyanathan *et al.*, 1997; Moncayo and Edman, 1999).

Not all mosquito species that show high vector competence for an arbovirus in the laboratory are vectors of that virus in the field, where other factors are also important. These include: sympatry with the vertebrate host species; occurrence with it in time; and an appropriate host preference. Species with vector competence that display these other characteristics are said to have **vectorial capacity**. Using the malariologists' concept of the basic reproductive number, Fine (1981) expressed vectorial capacity (V) quantitatively as the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding upon a single host in one day. Thus,

$$V = \frac{m\, a^2\, p^n}{-\ln p} \qquad (44.1)$$

where m is vector density per individual host, a is the average number of hosts bitten by one vector in one day, n is the extrinsic incubation period, and p is the probability of a vector surviving through a day.

Identification of the vectors involved in biological transmission of an arbovirus requires more than circumstantial evidence, and five criteria have been suggested as necessary to establish the role of a mosquito species as vector of a particular arbovirus: (i) records of isolation of the virus from that species in the field, with instances of minimum infection rates of sufficient value (Section 44.3.6.b); (ii) host preferences that include a known amplifying host; (iii) flight range and flight periodicity that overlap the distribution and temporal accessibility of the host; (iv) a seasonal period of host-feeding activity that coincides with that of disease incidence; and (v) laboratory demonstration of vector competence.

44.3 SEROLOGICAL AND MOLECULAR-GENETIC ASSAYS FOR ARBOVIRUSES

44.3.1 Introduction

Knowledge of arboviral diseases has been acquired since the beginning of the 20th century, and the development of techniques for the isolation of viruses and for the identification of virus species and strains has been fundamental to the growth of this knowledge. The findings from many early epidemiological investigations still provide key information, and some knowledge of the uses and limitations of assay techniques from all periods is necessary for a proper understanding of viral epidemiology.

Initially, diagnostic assays for arboviruses were developed for use in clinical medicine, but they became widely used in epidemiological investigations. For many years the tests were serological, concerning the constituents of serum, particularly antibodies. They were used both for diagnosis and to determine the characteristics of viruses, such as their distribution and prevalence rates. Not least valuable was the contribution of serological tests to arbovirus taxonomy, revealing degrees of relatedness between arboviruses. The tests were also used to 'subtype' arboviruses, revealing within individual arboviruses the numbers of distinctive serotypes, most now given the status of strains. Serological tests could provide epidemiologically important information. During epidemics of dengue fever, for example, from single samples of serum collected during the acute phase of infections, measurement of the avidity of immunoglobulin G could discriminate between cases of primary and secondary infection (i.e. infection for a second time but with a different serotype), which is an important indicator of the future severity of the disease (Matheus *et al.*, 2005).

Gradually, the availability of molecular techniques changed the situation, and in particular the sequencing of virus genomes made possible the rapid detection and identification of viruses. It also opened up the fields of viral phylogenetics (cf. Section 44.1.2.b) and of molecular epidemiology. The ability to distinguish the many strains of a

virus of different geographical origins, and to follow their movements through time, has contributed to epidemiological studies of dengue fever, Rift Valley fever and other arboviral diseases. However, in a reaction against the effective replacement of serological by molecular techniques, a Report of the American Committee on Arthropod-Borne Viruses noted that, although hundreds of viruses are entered annually in GenBank or other sequence databases, molecular data provide little or no phenotypic information, and asserted that 'the impact of this shift in emphasis has impaired studies of the biology of viruses'. Genotypic data from molecular studies and phenotypic data from serological studies are complementary, and both serological and molecular techniques should be used (Calisher *et al.*, 2001).

A key step in epidemiological investigations of an arbovirus is to establish which mosquito vectors or vertebrate amplifying hosts are infected; but that can be complicated if fragments of viral RNA or DNA are detected in tissues in the absence of infectious virus particles. Known examples from mosquito hosts are of sequences of Cell fusing agent virus and Kamiti virus which were integrated into the dsDNA genomes of wild-caught *St. albopicta* and *St. aegypti*. Evidence suggested an evolutionarily distant integration, but how integration into the genome occurred is difficult to determine (Crochu *et al.*, 2004; Cook *et al.*, 2006). A different example from a vertebrate host involved *Rabbit haemorrhagic disease virus* (*Lagovirus*, *Caliciviridae*), which is transmitted to rabbits by direct contact or fomites, or by oral secretions or anal excretions from bush flies. In rabbits that survived experimental infection with the virus, and in which neither infectious particles nor antigen could be detected, viral RNA was detected in leucocytes up to 7 weeks post-infection, and in liver, spleen and other organs for up to 15 weeks (Gall *et al.*, 2007). The relevance of these findings to arbovirus epidemiology is that in cases where such signs of infection have particular significance, as in overwintering mosquitoes, serological tests for the presence of infectious particles must also be used.

44.3.2 Antigens and antibodies

Particles of an arbovirus that have infected a vertebrate host are recognized by the host as antigens, and induce the production of specific antibodies, mostly glycoproteins of the immunoglobulin (Ig) family, which bind non-covalently and specifically with a corresponding antigen, usually counteracting its biological activity. In mammalian hosts of arboviruses, the antibody classes that are involved in most immune responses are IgM and IgG (immunoglobulins M and G). IgM generally becomes detectable 1–2 days after the onset of clinical symptoms, and remains detectable for 6–12 weeks. Specific IgG begins to increase in titre 1–2 weeks after the onset of symptoms, and remains detectable for many years, if not for life. Discrimination of IgM and IgG in serum samples proved effective in distinguishing recent from past infections of Japanese encephalitis virus (JEV) in pigs (Section 45.4.4.a), of RVFV in domesticated ruminants (Section 45.6.5.c), and of DENV in monkeys (Section 45.2.7).

Tests for arboviral infections in vertebrates require isolation of the virus or of antibodies from peripheral blood or target organs. Because the period of viraemia is short and free virus is rapidly bound by antibodies, isolation of the antigen can be difficult. Therefore, isolation and identification of antibodies is important in many cases. Serological tests can be tailored to address this problem.

Serological assays based on antigen–antibody interactions disclose the presence of specific antibodies in serum samples and measure their titre. Knowledge of the sensitivities and specificities of these assays is necessary to interpret the findings of investigations, whether early or more recent. During the phase of an infection when clinical symptoms are apparent, serological tests can reveal the nature of the infection even if free virus cannot be detected. They can also reveal long-past infections.

44.3.3 Amplification of viral isolates for serological assays

(a) Inoculation into mouse brain or birds' eggs

Samples are inoculated into the brains of 24–48-h-old laboratory mice, where the virus replicates, causing clinical symptoms and death. Most viruses attain a significantly higher titre in very young than in older mice. Positive samples can be titrated by intracranial inoculation of serially diluted samples into suckling mice to find the lethal dose (LD) for 50% of mice. Titres are expressed as '$\log_{10}$ mouse LD_{50} ml^{-1}' or 'SMIC (suckling mouse intracerebral) LD_{50} ml^{-1}'. Virus identity can be confirmed by immunofluorescence staining of brain tissue. Relatively pure preparations of virus can be obtained from the brain tissue, but sometimes a second passage is necessary (Clarke and Casals, 1958; Westaway and Blok, 1997). Serial dilutions of a sample can be inoculated into embryonated chicken or duck eggs and incubated.

(b) Inoculation into mosquitoes

Some arboviruses can be isolated most successfully by inoculation into mosquito cell cultures, which show no cytopathic effect, or into live mosquitoes, but isolation of a virus has to be confirmed by immunological means (Zuckerman *et al.*, 2004). When adult mosquitoes are used, serial dilutions of a sample are inoculated intrathoracically into either males or females of a species in which the arbovirus replicates and disseminates during the period of extrinsic incubation. *Stegomyia albopicta* has been used, but species of *Toxorhynchites* have the advantage of size and of safety, the females being non-haematophagous. When JEV was inoculated into *Toxorhynchites splendens* kept at 28 ± 1°C, virus particles could be detected in the salivary glands with fluorescent antibody 10–14 days later. Alternatively, mosquito larvae are inoculated intracerebrally, and brain tissue is examined later by immunofluorescence. With both adults and larvae, the larger species of *Toxorhynchites* (*Tx. splendens* and *Toxorhynchites amboinensis*) are easiest to inoculate. For dengue viruses, inoculation into mosquitoes proved more satisfactory than inoculation into mouse brain (Rosen and Gubler, 1974; Kraiselburd *et al.*, 1985; Gajanana *et al.*, 1995a, 1996).

44.3.4 Serological assays

(a) Haemagglutination and haemagglutination-inhibition assays

These assays provided much of the earlier information on arboviruses. Many viruses bear haemagglutinins – surface proteins that bind to erythrocytes. When added to a suspension of erythrocytes, these viruses bind and link many cells, forming a lattice which is detectable by eye. Used in early studies on JEV, this assay was rapid but rather insensitive. Antibodies that bind to haemagglutinins can block the binding of virus to erythrocytes, and that is the basis of the haemagglutination inhibition (HI) test. Serial dilutions of serum are incubated with a fixed amount of a virus and erythrocytes are added. If antibody specific for that virus is present, it will bind to haemagglutinins on the virus and agglutination will not occur. If agglutination occurs, the specific antibody is absent (Clarke and Casals, 1958; Flint *et al.*, 2000). In later studies, a modified HI test was used, in which some serum samples were first exposed to 2-mercaptoethanol (2-ME), which inactivated IgM but not IgG. Loss of activity in 2-ME-treated samples from a group of patients tested over a period of time could indicate the approximate date of first infection (Grossman *et al.*, 1973).

The historical importance of the HI test is apparent in the citations in Chapter 45 of its use in epidemiological investigations of EEEV (Eastern equine encephalomyelitis virus), DENV, JEV, LACV and RVFV.

(b) Direct immunostaining

This is used to visualize viral antigens present in tissues, or in cells from cell cultures, in which a

virus has been incubated. Histological sections, or other tissue or cell preparations, are treated with a specific antibody that is conjugated with an indicator. When the indicator is a fluorescent substance, the technique is an immunofluorescence assay.

(c) Enzyme-linked immunosorbent assay (ELISA)

This quantitative immunoassay can be modified to identify either arboviruses or the antibodies to arboviruses; its uses with antibodies are described below. Fuller details of ELISA techniques can be found in Volume 2, Section 39.1.4.

Using ELISA, a virus is allowed to bind by hydrophobic interactions to a carrier surface in the form of microtitre plates or beads of polyvinylchloride or polystyrene, and this antigen is used to capture a complementary antibody in a test solution. Previously, an enzyme had been covalently attached to the antibody (producing conjugated antibody), and the presence of the enzyme and its quantity are determined by the addition of a chromogenic substrate. Each enzyme molecule acts catalytically to produce many thousands of molecules of a detectable product, providing an amplified signal (Flint *et al.*, 2000).

The sensitivity of enzymic immunoassays can be enhanced by first amplifying any virus present in samples by culture in Vero cells within the wells of microtitre plates. After removal of the culture medium, the cells are fixed to the wells with 10% formalin, and a specific conjugated antibody is added (Graham *et al.*, 1986). Specificity can be enhanced by using monoclonal antibodies for the capture antibodies (Brown *et al.*, 2001). The assay was specific, rapid and economical when used for the detection of SLEV (St. Louis encephalitis virus) or WEEV (Western equine encephalitis virus) antigen in pools of mosquitoes or in bird tissue, but less sensitive than RT-PCR (Hunt *et al.*, 2002; Kramer *et al.*, 2002). A modification of the assay that used a proprietary dipstick produced results in less than 20 min and showed comparable sensitivity to the standard method when tested with SLEV and WNV (Ryan *et al.*, 2003).

(d) Antibody-capture ELISAs

These assays permit detection of specific antibodies present in serum. First, a particular viral antigen is adsorbed to the surface of wells in microtitre plates and serum samples are introduced into the wells. Antibody in the serum that is specific to the virus will bind to the immobilized antigen. The antigen–antibody complexes that form can be detected by adding to the wells a second antibody that is directed against a general epitope on the first antibody and also conjugated with an enzyme. A chromogenic substrate is added to the wells and its product quantified.

When used as the second antibody, monoclonal antibodies can distinguish between IgM and IgG, and they are used in IgM- and IgG-capture ELISAs to detect those immunoglobulins in serum (Kudesia, 1998; Flint *et al.*, 2000). IgM-capture ELISA permitted rapid diagnosis of infections with YFV (Saluzzo *et al.*, 1985), and provided a highly specific assay for JEV that was free of the problems of cross-reactivity with other flaviviruses shown by IgG (Endy and Nisalak, 2002). The IgM antibody ELISA (MAC ELISA) for cerebrospinal fluid was the most sensitive and specific method for serodiagnosis of JEV in human patients (Innis, 1995b). A modification in which IgM capture antibody is dotted on to a nitrocellulose membrane, and positive results are visible as a colour change, provides an assay suitable for field use that distinguishes between JEV and DENV (Solomon *et al.*, 1998). In cases of infection with Rift Valley fever virus, anti-RVFV IgG antibodies appear in human serum about day 4 and may remain at high titre indefinitely, providing a long-term indication of prior infection but with no indication as to when. In contrast, anti-RVFV IgM antibodies appear around day 5, are absent in 50% of patients by day 45, and are undetectable by 4 months after infection (Woods *et al.*, 2002).

(e) Indirect enzyme immunoassay

Immunization of a goat with the IgG fraction from pooled sera obtained from bird species rep-

resentative of a number of different orders (e.g. Passeriformes, Galliformes, Columbiformes and Anseriformes) induces production of polyclonal antibodies that react with serum proteins (including antibodies) from many bird species. Polyclonal antibodies obtained in that way are conjugated with horseradish peroxidase. The assay was first used to screen for anti-WEEV or anti-SLEV antibodies in serum samples from wild birds. With slight modification, it might be suitable for screening sera from most bird species for pathogens that induce antibody production. With appropriate discriminating titres, this assay was considered suitable for initial screening of field samples of sera from birds (Chiles and Reisen, 1998).

(f) Epitope-blocking ELISA

Immunodominant epitopes on viral surface proteins provide targets to which monoclonal antibodies can bind. In practice, the wells of microtitre plates are coated with a selected antigen; next, the test serum is added, and after a period of incubation a monoclonal antibody is introduced. Test sera, if positive, block binding of a homologous monoclonal antibody to the antigen. This assay was developed to distinguish between antibodies to two flaviviruses with a close antigenic relationship, namely Murray Valley encephalitis virus and Kunjin virus (Hall *et al.*, 1995). It can be used to test sera from any host species for the presence of a particular antibody. Blitvich *et al.* (2003) used it to detect WNV-specific antibodies in sera from a wide range of bird species.

(g) Virus-neutralization tests

To penetrate host cells, viruses first bind to specific molecules on the cell surface. Prior binding to such molecules by neutralizing antibodies blocks binding by virus particles and prevents invasion. Neutralization tests (NTs) test for the presence in serum of a virus-specific antibody that is able to neutralize (block) the infection and replication of a virus in a cell-culture system. Usually, neutralization tests were conducted by mixing dilutions of serum with virus, incubating, and then assaying for any remaining virus (i.e. virus that could infect and kill cultured cells or that could infect and replicate in embryonated eggs or whole animals). The end point was the highest dilution of the serum that had blocking effects. The results can have biological relevance as the production of neutralizing antibodies in response to a viral infection is important in establishing immunity (Flint *et al.*, 2000; Endy and Nisalak, 2002; Buckley *et al.*, 2003).

The 'plaque reduction neutralization test' (PRNT) is a modification of the neutralization test. Isolates from host tissue or serum are incubated with cells from vertebrate cell lines (e.g. Vero or BHK), or mosquito cell lines (e.g. Aa23 from *Aedes aegypti* embryonic tissue). Cells from such sources are allowed to form monolayers on the wells of microtitre plates while, separately, aliquots of diluted virus are incubated with diluted serum. The virus/serum suspension is added to wells and incubated, after which it is replaced with c. 1% agarose (to reduce spread of the virus) in culture medium and inactivated calf serum, and the incubation is continued for some days before addition of fixative. If the virus has spread only to neighbouring cells it forms a plaque of dead cells which, after fixation and staining, allow the plaques to be visible as clear areas in the monolayer. Each plaque is graded as one plaque-forming unit (PFU), and represents one virus particle or more depending on the extent of aggregation. It is an advantage of the PRNT test that it detects only infectious virus particles.

44.3.5 Molecular-genetic diagnostics

Molecular-genetic techniques often now replace serodiagnostic methods, although currently they provide little information on the phenetic characteristics of virus species. They are used principally in the detection and identification of arboviruses isolated from their hosts, and in the provision of sequence data for taxonomic purposes. Sequence

data are also used in determining the phylogeny of higher taxa. Methods have been developed to detect viral nucleic acids from among a vast excess of host sequences, when present within infected cells or tissues. By other methods it is possible to amplify genomic constituents and to detect particular nucleic-acid sequences. Assays that start with amplification are time-efficient and highly sensitive.

(a) Northern blotting

This is a form of blotting in which the primary electrophoresis is performed with RNA, which is subsequently hybridized to radioactive DNA. The term distinguishes the process from Southern blotting, which is performed with DNA. In Northern blotting, RNA extracts are digested with a restriction endonuclease to produce fragments of RNA. These are fractionated by gel electrophoresis, denatured within the gel, and transferred to a membrane to which they bind. The bound sequences are then analysed by hybridization to complementary radioactive or biotinylated single-stranded DNA probes; the hybrids are detected by autoradiography or colour change, respectively (Rieger *et al.*, 1991; Flint *et al.*, 2000). Northern blotting has been largely superseded by other molecular techniques.

(b) Reverse transcriptase-polymerase chain reaction (RT-PCR)

This process involves the enzymes reverse transcriptase and DNA polymerase. Because most arboviruses have an RNA genome, often the target RNA in a sample is converted to cDNA (complementary DNA) using reverse transcriptase, and the DNA is amplified by polymerase chain reaction. Reverse transcriptase is an RNA-directed DNA polymerase able to copy single-stranded RNA sequences into complementary double-stranded DNA. Polymerase chain reaction is an enzymatic technique that allows the amplification of a region of DNA that is situated between two convergent primers. By coupling RT and PCR, up to 100,000-fold amplification of the DNA may be attained. The amplified DNA sequences can be identified by hybridization to specific oligonucleotide probes representing particular virus strains or particular genera (Rieger *et al.*, 1991; Smith *et al.*, 1997).

Modification of RT-PCR by the use of TaqMan primers provided greater amplification, specificity and speed of response in tests for viral RNA in mosquito pools, or in tissue from their vertebrate hosts (Lanciotti *et al.*, 2000; Lanciotti and Kerst, 2001; Lambert *et al.*, 2003). During epidemics of West Nile fever, the TaqMan RT-PCR assay gave positive responses to 98% of tissue samples from dead birds shown to have been infected with WNV, and gave positive responses to 100% of brain tissue samples and to 57% of cerebrospinal fluid samples from seropositive humans (Lanciotti *et al.*, 2000). In a 4-year field study, a system named RAMP produced fewer false positives for WNV than the TaqMan assay, but was less sensitive. It was recommended as a valuable tool when used to augment RT-PCR-based WNV surveillance programmes (Williges *et al.*, 2009; Section 45.7).

The RT-PCR test involves extraction of nucleic acids by procedures that destabilize cellular structure, with release of cell contents. Then, addition of phenol denatures proteins so that, after centrifugation, the liquid phase contains nucleic acids which can be purified further. Because RT-PCR tests detect infectious particles and non-infectious virus particles and free nucleic acid, positive results do not indicate whether or not viable virus particles are present. For that purpose the serological PRNT test described earlier is used.

44.3.6 Field evaluations

(a) Limitations of methodology

Before it became possible to search for viral RNA or DNA, it could be extremely difficult to isolate virus for diagnostic purposes from hosts that were not amplifying hosts. This can be illustrated by a study of human cases of confirmed or probable JEV infection in Thailand. Plasma samples had

been obtained from 49 cases, of which 34 were non-fatal and 15 fatal, but none of those samples yielded virus. Of 30 samples of cerebrospinal fluid from non-fatal cases none yielded virus, but five of 15 samples of cerebrospinal fluid from fatal cases did yield virus. Fresh brain specimens obtained at autopsy from seven fatal cases yielded virus in every case (Leake *et al.*, 1986a).

In historical time, and continuing today, the distributions of a number of flaviviruses have come to overlap, e.g. those of dengue virus, Japanese encephalitis virus, West Nile virus and Murray Valley encephalitis virus. In such situations, diagnostic assays must be both highly sensitive and highly specific.

Usually when mosquito populations are infected with an arbovirus, the proportion of infected individuals is exceedingly low. To detect the presence of an arbovirus, and to determine its infection rate in the wild mosquito population, it may be necessary to capture and screen thousands or even hundreds of thousands of individuals. After the capture of mosquitoes, their separation by species, and the removal of recently blood-fed females, the collection of a particular species is separated into a number of 'pools', and each pool is tested for the virus. The pools may contain a constant number of mosquitoes or, owing to circumstances, may vary in size (e.g. 25, 50, 100 mosquitoes). To estimate the proportion of infected individuals in a collection, it is necessary that at least a few pools are negative even when infection rates are high, and that appropriate statistical methods are used. The purpose of pooling samples is to reduce the effort and cost of testing.

Gu and Novak (2004) explored mathematically what is apparent intuitively: that the lower the infection rate in a population, the lower is the probability of detection of infected individuals. For simplicity, they assumed that infected and non-infected mosquitoes were captured with equal probability, and that the arbovirus test was highly sensitive and 100% reliable in specificity. The pools tested had only two possible states, infected or uninfected – the characteristic of a binary variable. Therefore, based on the binomial distribution, the probability of detection (P) of any infected individuals in a pool of N mosquitoes could be calculated from

$$P = 1 - (1 - r)^N \tag{44.2}$$

where r is the infection rate. For any sample N, the smaller r is, the smaller is P. Given infection rates in two populations of 1 and 5 per 1000, the probabilities of detecting infected individuals in a sample of 500 mosquitoes would be 0.39 and 0.91, respectively. With small sample sizes (total <300), the probability is >0.05 that no infected individuals will be detected even when the infection rate is relatively high, e.g. 5 per 1000. For any given infection rate, it is possible to calculate the smallest sample size needed for a specified probability of detection by manipulating Eqn. 44.2, such that

$$N = \log(1 - P) / \log(1 - r) \tag{44.3}$$

When infection rates are low, large numbers of mosquitoes are needed to obtain a high probability of detection (Figure 44.3). For example, when the infection rate is 1 per 1000, the numbers of mosquitoes needed for detection probabilities of 0.5, 0.8 and 0.9 are 693, 1609 and 2301, respectively.

Typically, in any large geographical area, the occurrence of an arbovirus in populations of its

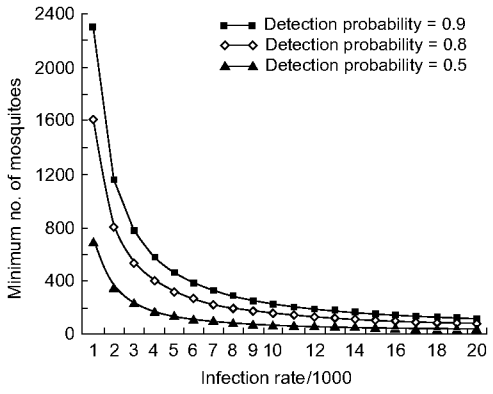

Figure 44.3 Minimum numbers of mosquitoes required at different infection rates for detection probabilities of 0.5, 0.8 or 0.9. (From Gu and Novak, 2004.)

mosquito host shows spatial and temporal variability. Therefore, combining samples obtained from widely separated locations, or collected over extended periods of time, defeats attempts to assess detection and infection rates. Two approaches to this problem have been proposed. From published findings on the occurrence of St. Louis encephalitis virus and West Nile virus in their mosquito hosts, Gu and Novak (2004) recommended that for detection of arbovirus transmission generally, sampling should be intensive in areas where human/mosquito contact is common or where, historically, infected mosquitoes have been found. The presupposition of mosquito distribution inherent in that approach carries a risk of error that is avoided in an alternative recommended by Reisen and Lothrop (1999). Among five sampling strategies that were evaluated with *Culex tarsalis* populations in California, a 'stratified random design' provided the most accurate and precise simulation.

Turell *et al.* (2002) identified risks that are inherent in sampling procedures, especially when mosquitoes are grouped in pools for screening. (i) An infected mosquito might be misidentified and placed in the wrong pool. In fact, a single leg from a mosquito infected with, for example, West Nile virus can contain up to 10,000 plaque-forming units of virus. (ii) The transfer of infected haemolymph on to instruments used to handle specimens could lead to contamination. (iii) Where pools include blood-engorged mosquitoes, a positive result might arise from virus in a blood meal rather than from an infected mosquito. Because of these problems, the authors suggested that the presence of virus in only a single pool, or even in a small number of pools of a species, may not be sufficient to implicate that species in the transmission process.

(b) Estimation of infection rate

Traditionally, after the capture of mosquitoes and their separation by species, the catch of a particular species was divided into a number (n) of pools, all containing a constant number (m) of mosquitoes, and each pool was tested for the presence of a virus. A positive test meant that at least one of the m mosquitoes in a pool carried the virus. Assuming that non-infected and infected mosquitoes had been randomly assigned into pools, the number of infected mosquitoes in a pool was a binomial random variable with potential values of 0, 1, ..., m. A simple but crude measure of prevalence rate was given by the 'minimum infection rate' (MIR), the estimated lower limit of the true infection rate expressed as the number of infected mosquitoes per thousand tested. Thus

$$\mathrm{MIR} = Y/N \times 10^3 \qquad (44.4)$$

where Y is the number of positive pools and N is the total number of mosquitoes tested. When pool size is constant, $N = mn$. The validity of MIR as a measure of infection rates depends on the assumption that there is only a single infected individual in a positive pool. When infection rates are low or pool size is small, MIR provides a good estimate of the true infection rate because the chance of more than one infected individual being present in a positive pool is very low. If the pool size is large or many specimens are infected, MIR may seriously underestimate the true infection rate. The MIR is not useful when pool size varies and a number of small pools are included in the analysis.

An alternative and statistically sound approach, namely 'maximum likelihood estimation', has been examined for suitability (Chiang and Reeves, 1962; Bhattacharyya *et al.*, 1979; Walter *et al.*, 1980). This approach makes fuller use of the available data, and relaxes the assumption underlying MIR that only one infected individual is present in a positive pool. Analysis of data by these procedures yields a 'maximum likelihood estimate' (MLE), i.e. the infection rate most likely to generate the test results given a probabilistic model (binomial distribution). For constant pool size, the maximum likelihood estimate is given by

$$\mathrm{MLE} = 1 - (1 - Y/X)^{1/m} \qquad (44.5)$$

where Y is the number of positive pools, X is the total number of pools, and m is pool size (Chiang

and Reeves, 1962, as expressed by Gu *et al.*, 2003). For given populations, values of MIR (Eqn 44.4) and MLE (Eqn 44.5) are similar when infection rates are low and pool sizes are small, but they can differ significantly when infection rates are high.

When pool size is variable, no analytical solution of MLE is available and iteration procedures must be used (Walter *et al.*, 1980, Gu *et al.*, 2003, 2004). The likelihood function (L) is

$$L(p \setminus m, r) = \prod_{i=1,\ldots,m} (1-p)^{n_i} \prod_{i=1,\ldots,r} (1-p)\, n_i' \qquad (44.6)$$

where p is the infection rate; m and r are the numbers of negative and positive pools, respectively; and $m = (n_1, \ldots, n_m)$ and $r = (n'_1, \ldots, n'_m)$ are the arrays of pool sizes for the negative and positive groups of pools, respectively. Both the point and interval estimates of p can be obtained by numerical iterations based on Eqn 44.4 (Walter *et al.*, 1980). A computer program (MLE-IR) for calculating the MLE of infection rates and their confidence levels is available from the US Centers for Disease Control and Prevention (CDC) (Biggerstaff, 2003). In a study designed to compare the MIR and MLE approaches for estimation of infection rates of West Nile virus (WNV) in *Culex pipiens* and/or *Culex restuans*, females that had been captured in oviposition traps near Chicago were batched in pools of 9–50 and tested for WNV. During August 2002, when transmission of WNV at that location was at its peak, the MLE varied around 60/1000, while the MIR was 30/1000 or less. During September 2002, when infection rates were 10–20/1000, there was little difference between the MIR and MLE estimates (Gu *et al.*, 2004).

A simulation study conducted on a hypothetical mosquito population showed that both MIR and MLE can be inaccurate when almost all pools are positive, whether due to high infection rates or large pool size (Gu *et al.*, 2004). Under such circumstances, reduction in pool size can improve accuracy. Estimates of infection rates were compared between varied-size pools (5, 10, 20, 30, 40 and 50 females) and pools of constant size (50 females). Hundreds of replications were run, and means of MIR and MLE were calculated (Figure 44.4). When infection rates did not exceed 10/1000, both MIR and MLE corresponded closely with the true infection rate, whether pool size was varied or constant. At 30/1000, the MLE and MIR were slightly above and below the true rate, respectively. From infection rates of 40/1000 to 70/1000, MLE with the constant-size pools of 50 was excessively high, but MLE with varied-size pools tracked only slightly above the true rate. MIR with both constant- and varied-size pools tracked at a low and increasingly incorrect rate (Gu *et al.*, 2004). This analysis suggested that at infection rates of 10/1000 or less, both MIR and MLE, with constant or varied pool size, provide accurate estimates, but that at higher infection rates the most accurate results would be obtained with varied-size pools coupled with MLE.

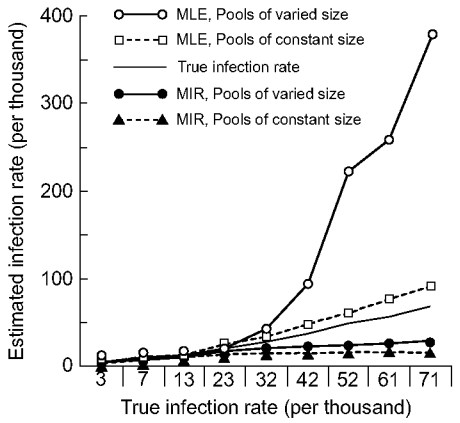

Figure 44.4 Calculation of rates of infection with West Nile virus in a hypothetical population of *Culex* spp., as determined by the minimum infection rate (MIR) and maximum likelihood estimate (MLE) of infection rate, using pools of constant or varied size. (After Gu *et al.*, 2004.) Varied pool size: 12 pools, two each of 5, 10, 20, 30, 40 or 50 mosquitoes; total 310 mosquitoes. Constant pool size: 12 pools of 50 mosquitoes; total 600 mosquitoes. Results were obtained by numerical iterations based on Eqn 44.3. In this graph, the sequence of true infection rates on the *x*-axis is not regular, consequently the curve representing the true infection rate is not a smooth curve.

44.4 MODES OF TRANSMISSION TO VERTEBRATE HOSTS

44.4.1 Transmission by blood-feeding mosquitoes

Overwhelmingly the most common way in which vertebrates become infected by mosquito-borne viruses is through the bites of infective females. Each successful act of blood feeding comprises four phases: (i) exploration of the skin surface; (ii) probing the skin with the mouthpart stylets while ejecting saliva; (iii) imbibing blood; and (iv) withdrawal of the stylets. Females spend a variable length of time probing before locating a suitable blood vessel, and it is during this phase, to facilitate location of a blood vessel, that most saliva is discharged. When her stylets have penetrated a small arteriole or venule in the skin, a female imbibes blood by one or other of two processes. If the tips of her stylets are flexed and the stylets pass along the blood vessel, she imbibes blood from within the vessel. If the stylets lance a blood vessel, passing through it, and are then partially withdrawn, blood pressure forces blood from the damaged vessel into skin tissue, producing a small pool or haematoma, and the female imbibes blood from that as it continues to fill. These processes are termed 'vessel feeding' and 'pool feeding' respectively (Volume 1, Section 11.3.3).

Arboviruses pass from the salivary glands of their mosquito host to the vertebrate host carried in saliva, and most transfer of virus into host skin occurs during the probing phase when saliva is discharged. When mosquitoes infective with the malaria parasite *Plasmodium* feed, sporozoites can be found later within the blood bolus in the mosquito midgut, having been reingested by the mosquito. That as many sporozoites could be reingested as remained in the skin (Kebaier and Vanderberg, 2006) shows the effectiveness of transmission of infectious agents by engorging females to have been much less than was believed.

Experiments were undertaken with laboratory mice as hosts to find whether virus is inoculated extravascularly into tissue or directly into the circulating bloodstream. The virus/mosquito combination was Rift Valley fever virus (*Bunyaviridae*) with the putative vector *Cx. pipiens*. Mosquitoes were inoculated intrathoracically with virus and 6–8 days later allowed to probe the distal one-third of the tails of mice for 20–30 s. At intervals of 1, 5, 10, 60, 120 or 360 min after the end of probing, the tails of test mice were severed at the midpoint and their length of survival was recorded. Mice whose tails were not severed served as positive controls. Among mice probed by mosquitoes infective with RVFV, severance of the distal half of the tail within 5 or even 10 min of their being probed significantly increased the length of survival compared with mice whose tails remained intact (Figure 44.5). Only 28% of mice whose tails were severed ≤ 5 min after probing died, compared with 92% of those whose tails remained intact. A median of $10^{2.5}$ PFU was recovered from the tails of mice that had been severed ≤ 10 min after probing. Because many of these mice did not become infected with RVFV, probably that amount represents the average amount of detectable virus that the mosquitoes inoculated while probing. At each time interval studied, the amount of virus recovered from the tails of mice that survived was less than that from the tails of those that later died (Turell and Spielman, 1992).

Similar results were obtained with two other viruses: St. Louis encephalitis virus (*Flaviviridae*) with the putative vector *Cx. tarsalis*; and Venezuelan equine encephalitis virus (VEEV) (*Togaviridae*) with the putative vector *Ochlerotatus taeniorhynchus*. Among mice inoculated with SLEV, only 3% of mice with intact tails survived for 5–6 days, compared with 34% of those whose tails were severed at the midpoint ≤ 10 min after the end of probing ($p < 0.0001$). Among mice inoculated with VEEV, only 4% with intact tails had survived at 43 h after the end of probing; in contrast, 37% of mice whose tails were severed ≤ 10 min after probing were alive at 48 h after being probed ($p < 0.001$) (Turell *et al.*, 1995).

Mosquitoes can inoculate >3000 LD_{50} of RVFV or SLEV, therefore all of the mice with severed tails would have died if even 0.1% of the inoculum had entered the circulating blood directly or travelled 2–5 mm in lymph to pass the site of

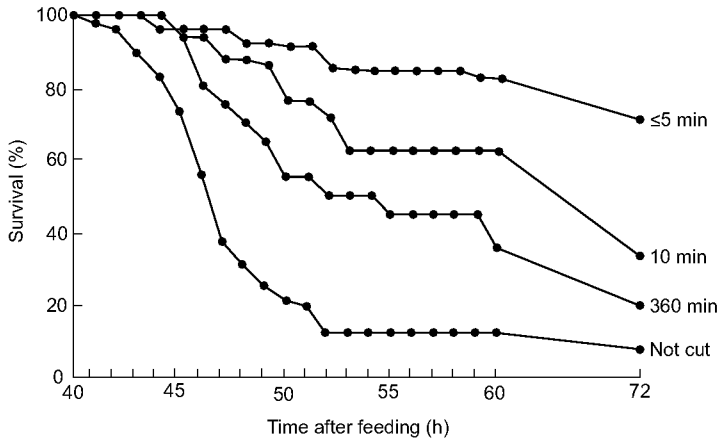

Figure 44.5 Survivorship curves of mice viraemic with Rift Valley fever virus as a result of *Culex pipiens* having probed the distal part between their tails shown in relation to the duration of the period between the end of probing (time after feeding) and severance of the tails at the midpoint. (After Turell and Spielman, 1992.)

severance. The investigators concluded that, in most cases of blood feeding by infective mosquitoes, the viruses are inoculated extravascularly during the probing phase of blood feeding (Turell and Spielman, 1992; Turell *et al.*, 1995).

44.4.2 Transmission on the mouthparts of vectors

The explosive spread of arboviral diseases through populations of hosts that sometimes occurs has led to the suggestion that 'mechanical transmission' may be responsible, i.e. transmission in the mouthparts of vectors. In theory, any mobile blood-sucking arthropod can transmit an arbovirus mechanically under favourable conditions, but not necessarily to an extent that is epidemiologically significant. Turell (1988) suggested that the requirements for mechanical transmission are a virus that is stable under environmental conditions and that induces a high-titred viraemia, and vertebrate hosts that are highly susceptible. Proof of mechanical transmission by mosquitoes in the field has not been obtained for any arbovirus. (Examples of the mechanical transmission of pox viruses are described in Section 43.3.)

Mechanical transmission between small experimental hosts has been demonstrated in the laboratory. One example was of Eastern equine encephalomyelitis virus (EEEV) transmitted to chicks by Oc. *triseriatus*. Biological transmission cannot occur before completion of the extrinsic incubation period, so we note first that an extrinsic incubation period of 9 days was determined for EEEV in Oc. *triseriatus* (not a natural host) when kept at 27°C. Virions were first seen in the mosquitoes' salivary glands 9 days after infective feeding, at which time some of the mosquitoes that fed on viraemic chicks could infect healthy chicks by bite (Whitfield *et al.*, 1971). Previously, EEEV transmission had been measured in a batch of Oc. *triseriatus* that probed viraemic chicks and then, after an interval of 3, 6, 9, 13 or more days of incubation, probed on healthy chicks. When the mosquitoes were kept at 21°C, 60% transmitted on the third day, 14% on the sixth, and 4% on the ninth. Subsequently, the rate of transmission increased again, reaching 33% on the 13th day, 57% on the 17th and 76% on the 33rd. Chamberlain and Sudia (1955) surmised that, for mosquitoes kept at 21°C, any transmission was mechanical for up to at least 9 days after they had

probed viraemic chicks, and that the increased rates from day 13 onwards indicated virus replication and biological transmission. Further experiments, including the use of infected pins, supported this conclusion (Table 44.3) (Chamberlain and Sudia, 1961). When females of *Ochlerotatus vigilax* were placed with suckling mice infected with Ross River virus, and transferred when partly engorged to probe on uninfected suckling mice, the virus was transmitted mechanically in 15% of cases (n = 20) (Kay, 1982).

The speed with which infections with Rift Valley fever virus (RVFV) spread through flocks of sheep in South Africa led to the suggestion that biological transmission by mosquitoes might be augmented by mechanical transmission by biting flies (Jupp *et al.*, 1984). In the laboratory, RVFV was transmitted to uninfected hamsters by mosquitoes which, 0–5 min earlier, had taken an interrupted feed on hamsters with a high-titre viraemia. With three species of mosquitoes, the transmission rates were: *St. aegypti* 15% (n = 52); *Oc. taeniorhynchus* 15% (n = 20); *Cx. pipiens* 8% (n = 60). Mechanical transmission of RVFV was obtained also with species of *Stomoxys*, *Lutzomyia*, *Culicoides* and *Glossina*. With *Glossina morsitans*, the rate of transmission was positively correlated with extent of viraemia and negatively correlated with time between the two feeds. RVFV was mechanically transmitted to two lambs by *Cx. pipiens* that had fed on viraemic hamsters, one lamb being exposed to 50 and the other to 13 mosquitoes. Single tsetse flies also transmitted the virus to lambs (Hoch *et al.*, 1985).

Table 44.3 Mechanical transmission of Eastern equine encephalitis virus from infected to uninfected chicks by probing *Ochlerotatus triseriatus* and by pinprick. (From Chamberlain and Sudia, 1961.)

Interval between infecting and transmitting probes (h)	*Successful transmissions*	
	Mosquitoes (%)	*Pins (%)*
0	100 (10) *	100 (5) *
1	90 (10)	100 (5)
4	70 (10)	100 (10)
20	60 (20)	40 (10)
70	20 (20)	10 (20)

* Number of probes or pinpricks (in parentheses).
Females of Oc. *triseriatus* probed briefly on a viraemic chick without taking visible blood, and probed again from 0 to 70 h later on healthy chicks. Pins were inserted c. 6 mm under the skin of a viraemic chick and later reinserted into healthy chicks. Mosquitoes and pins were kept at c. 27 °C and 75% relative humidity. There was no significant difference between mosquitoes and pins in effectiveness of transmission.

44.4.3 Contact with infectious tissue or blood

Berry and Kitchen (1931) noted the presumed cause of 32 'accidental infections' with yellow fever virus in laboratories that had been reported from around the world between late 1927 and early 1931. Eliminating 11 of the reported observations, which were from the tropics, where the possibility that the infections had been contracted outside the laboratory could not be discounted, the findings were: (i) contact with infectious monkey blood or tissue, 15 cases; (ii) contact with infectious mouse brain, two cases; (iii) handling infected animals, four cases. The severity of the infections ranged from mild to fatal.

There have been many observations consistent with the passage of Rift Valley fever virus directly from infected animals to humans, either during slaughter or during the post-mortem examination of diseased animals. As one example, analysis of the case records of over 50 farmers and farm workers who fell ill with RVF during an epidemic in South Africa revealed that all had had close contact with sick sheep or cattle or with meat, and that most had cut open and handled the viscera of sheep. All veterinary surgeons who had carried out post-mortem examinations on infected sheep contracted RVF. Several housewives contracted the infection, it was thought from handling infected mutton. There was no evidence of infection spreading from patients to individuals coming into contact with them (Joubert *et al.*, 1951).

Contact with aborting female ruminants carries a particular risk. For example, during an RVF epidemic in southern Mauritania, almost every human case from two villages was associated with

animal abortion (Jouan *et al.*, 1989a). In some of these cases the virus might have been transferred in aerosol (see Section 44.4.4) but, probably, direct contact with infected tissue or blood was also a means of transfer. Rubbing one or two drops of blood, obtained from rhesus monkeys during the early stages of viraemia with yellow fever virus, on to intact skin of the abdomen of other rhesus monkeys led to their infection (Bauer and Hudson, 1928).

44.4.4 Transmission in aerosols

It is not only viral respiratory infections that are contracted via the respiratory tract, but also some generalized infections, such as measles and smallpox, which also are caused by viruses. After the virus has replicated, some virus particles escape from the respiratory tract in an aerosol of liquid droplets during talking, coughing or sneezing. Simplifying one definition of the term, an 'aerosol' is here taken to be 'a suspension of fine particles dispersed in air'. Aerosols may be inhaled into the respiratory tract and lungs, or may fall on to the conjunctiva, where the virus replicates and is carried by natural drainage to the throat and respiratory tract. The size of the particles is important: particles >10 μm diameter quickly fall to the ground, and particles <0.3 μm diameter dry very quickly, inactivating any virus they contain (Dimmock *et al.*, 2001).

Inhalation of aerosol particles containing RVFV was thought to have been one mode of transmission during the 1977 epidemic of Rift Valley fever in Egypt, which caused many infections and fatalities. A proportion of the human infections was ascribed to the slaughter of sick animals, for consumption, by the traditional method of throat cutting. During a field trip by seven members of a US Naval Medical Research Unit, six team members were present during the slaughter of a sheep, but had no physical contact with the sheep. Three days later all developed virologically proven RVF, but the seventh member, who was not present in the room, remained healthy. The two villagers who slaughtered the sheep also became ill. A serum sample from the sheep had an RVFV titre of 10^{10} SMIC LD_{50} ml^{-1}. The infections were ascribed to inhalation of virus in blood droplets discharged from the dying sheep (Hoogstraal *et al.*, 1979).

Aerosols of RVFV and of yellow fever virus (YFV) could be produced in the laboratory by forcing dilutions of the virus in broth through a spray system capable of emitting particles of 4 μm median diameter. Measurements of the decay rates for RVFV aerosols showed RVFV to be very stable at 24°C and either 50% or 85% relative humidity. After 1 h in aerosol form, a substantial proportion of the original airborne virus was still active: the biological decay rate was estimated to be 1.14% per min. The decay rate of YFV was rather faster (Table 44.4). All rhesus monkeys exposed to aerosols of RVFV survived; however, even at the lowest dose tested, all became viraemic for a period of 4 to 6 days. Among the 18 monkeys exposed to YFV all became viraemic; between days 7 and 13 post-exposure, 12 of the 18 monkeys died (Miller *et al.*, 1963).

44.5 VENEREAL TRANSMISSION

44.5.1 Introduction

Venereal transmission is the passage of infectious agents carried in semen from vertically infected males to females during coition. It is a form of horizontal transmission (Section 41.2.2). Male mosquitoes capable of transmitting an arbovirus during coition can themselves have become infected only through vertical transmission. Venereal infection can contribute to the perpetuation of an arbovirus in two ways: (i) by increasing the number of infected females in a population that can transmit to amplifying hosts; and (ii) by infecting females that survive long enough (effectively for two gonotrophic cycles) to lay vertically infected eggs.

Venereal transmission has not been observed between mosquitoes in the field, but it has been demonstrated in the laboratory with species of *Ochlerotatus*, *Stegomyia* and *Halaedes*, and arboviruses of the families *Bunyaviridae*, *Flaviviridae* and *Togaviridae*.

Table 44.4 Properties of aerosols of Rift Valley fever virus and yellow fever virus, and their effects on rhesus monkeys. (From the data of Miller *et al.*, 1963.) Sixteen monkeys were exposed to different inhaled doses of RVFV aerosol, and 18 to different inhaled doses of YFV aerosol. Characteristic results from exposures to the lowest doses are shown here.

A. Properties of aerosols

	Aerosol retrieval after 4 min		*Decay rate*	
Source of virus	*Mean (%)*	*95% conf. limits*	*Mean (% min)*	*95% conf. limits*
Rift Valley fever virus				
Lamb plasma	9.25	2.4–36.1	1.14	0.9–2.3
Hamster kidney cell culture	9.42	2.4–36.8	2.95	0.5–5.3
Human liver cell culture	19.40	4.9–75.7	3.62	0.9–6.2
Yellow fever virus				
HeLa cell culture	67.20	35.1–128	5.90	3.6–8.2

B. Effects of inhalation on monkeys

Monkey number	*Inhaled dose (MIC LD_{50})*	*Plasma dilution for viraemia test*	*Monkeys with viraemia (%)*				*Survival, or day of death*
			Day 4	*Day 6*	*Day 8*	*Day 10*	
Rift Valley fever virus							
4603	145	10^1	20	100	90	30	Survived
		10^2	50	100	100	10	
4600	76	10^1	80	100	50	20	Survived
		10^2	60	80	20	10	
Yellow fever virus				*Day 5*	*Day 7*		
5000	6.0	10^1		70	90		Day 10
		10^2		30	100		
4640	6.0	10^1		100	100		Survived
		10^2		30	22		

Particles of RVFV or YFV were harvested from lamb plasma or cell cultures (as indicated) and diluted in beef broth to titres measured as mouse intracerebral (MIC) LD_{50} ml^{-1}. Aerosols were produced by a spray system capable of emitting particles with a median diameter of about 4 μm. Measurements of aerosol recovery and stability were made with guinea-pig assays. First recoveries of aerosol, after 4 min, were expressed as percentage of the amount disseminated. Further measurements, at intervals, indicated the decay rate. Batches of nine or ten monkeys were exposed to each aerosol concentration, their heads being within a cloud chamber. To identify viraemic monkeys plasma samples were taken at intervals, diluted 10^1-fold or 10^2-fold in broth, and injected intraperitoneally into mice.

Loosely defined, for any population, the so-called 'venereal transmission rate' is the proportion of the female population that becomes infected by copulation with vertically infected males. However, a natural population that includes vertically infected males also includes vertically infected females, the venereal infection of which does not further enhance perpetuation of the infective agent. The term 'effective venereal transmission' relates to the proportion of the female population that is infected venereally and only venereally. The 'efficiency of venereal transmission' is the proportion of uninfected females that having mated with a vertically infected male becomes venereally infected. The 'rate' of

venereal transmission is the product of the proportion of adult males that are vertically infected and the efficiency of venereal transmission.

By definition, the transfer of a virus from a venereally infected female to her offspring is a form of 'vertical transmission' i.e. the transference of an infectious agent from a parent organism to its progeny.

44.5.2 Laboratory experiments

Most laboratory experiments have involved Oc. *triseriatus* as host species and La Crosse virus (LACV) (*Bunyaviridae*) as the infective agent.

(a) Methods and initial findings

In most cases, studies of venereal transmission start with the addition of a batch of males, some of which are vertically infected with an arbovirus, to a cage containing a batch of uninfected females. The proportion of the male population that was infected is determined after the period of mating. The proportion of the female population that becomes venereally infected may be determined immediately after mating by dissection and examination of the distal reproductive tract, or at a later date by virological tests. In cage experiments with more than one male it is not known how many females have been inseminated by more than one male, so the efficiency of venereal transmission cannot be determined. An alternative method is 'forced mating', when infected males are decapitated, held individually in forceps, and their genitalia are brought into contact with those of an uninfected female.

Venereal transmission was first demonstrated in mosquitoes when male Oc. *triseriatus*, of which 31% were vertically infected with La Crosse virus, were caged with non-infected females at a 1:5 ratio. A blood-meal source was provided during weeks one and three, and the males were removed after 10–12 days. Of females removed for examination 18–26 days after mating, 2.5% contained viral antigen. Fluorescent-antibody staining revealed viral antigen in organs other than the lower genital tract in a small number of the females. No transfer of virus occurred in the opposite direction when females infected with LACV were caged with uninfected males for up to 26 days. To check that this venereal transmission was not an artefact resulting from contamination, e.g. from male faeces or from a contaminated sugar source, male Oc. *triseriatus* parenterally infected with LACV were force mated with uninfected females, when the only interaction between them was contact of genitalia during copulation. Most of the females became infected. Within the first 10 min after forced mating, LACV was present in the females' bursa inseminalis, but by 6 h post-mating the number of females with virus in the bursa had declined to c. 50%, and it continued to decline thereafter. Eventually, these females had disseminated infections (Thompson and Beaty, 1978).

Use of fluorescent antibody to visualize antigen in adult male Oc. *triseriatus* vertically infected with LACV showed a weak response in the testes and the lumina of the vasa efferentia and seminal vesicles, but an incomparably stronger response in the contents of the accessory glands. Viral antigen was observed in fluid forcibly extruded from the male ejaculatory duct, and after the forced mating of vertically infected males with uninfected females viral antigen was detected in the contents of the females' bursa inseminalis. Viral antigen could not be detected in sperm, so virus that was transferred in semen was carried in the accessory-gland secretion (Beaty and Thompson, 1976; Thompson and Beaty, 1978).

Venereal transmission of a flavivirus was achieved when male *St. albopicta* parenterally infected with dengue virus were caged with uninfected females for 2 weeks. The proportion of females venereally infected was 3.2% for males inoculated 7 days before mating, and 27.4% for males inoculated 14 days before mating. Females venereally infected with dengue virus could transmit the virus vertically to their offspring, apparently by transovum transmission (Section 44.6.1.b). Sexual transmission of virus was in one direction only. Females inoculated with DENV

and mated 14 days later did not transmit the virus to males, but could transmit it vertically to their progeny (Rosen, 1987a). Venereal transmission of Sindbis virus (*Togaviridae*) occurred when male *Halaedes australis* that had been parenterally infected were force mated with uninfected females, and occurred whether or not sperm were transferred (Ovenden and Mahon, 1984).

(b) Relationship to blood feeding

Certain experimental results led to the claim that the efficiency of venereal transmission is affected by the relative timings of mating and blood feeding. Among female Oc. *triseriatus* (n = 139) that blood fed 2 days after being caged with males parenterally infected with LACV, only 2.1% transmitted the virus by bite during the period 8–40 days post-mating. In contrast, among females (n = 42) that had engorged 6 days before being caged with infected males, 5% could transmit the virus to mice by bite on days 4–7 post-mating, and 32% (n = 37) could do so on days 8–17 post-mating (Thompson, 1979).

Another study threw doubt on the significance of the timing of blood feeding in relation to mating. Batches of adult male Oc. *triseriatus* used in the study were the F_1 progeny of females that had been intrathoracically inoculated with LACV, and when eventually tested showed vertical transmission 'rates' of 32–79%. Batches of uninfected females were caged with batches of males (sex ratio 1:1) for 7-day periods. The females were blood fed either 6–8 h before being caged with males or immediately after the 7-day mating period. In the batches of females blood fed 6–8 h before being caged with males (of which 44–79% were vertically infected), 30–53% became venereally infected. In the batches of females blood fed after being caged with males (of which 32–67% were vertically infected) 16–57% became venereally infected (Patrican and DeFoliart, 1987).

Scholl *et al.* (1979a) reported that 45% of wild Oc. *triseriatus* females collected while biting had not yet been inseminated.

(c) Transmission by venereally infected females

Venereally infected females can transmit the virus vertically to their offspring and also horizontally to hosts on which they feed.

Investigators have reported delays of some days between females becoming venereally infected and laying infected eggs. Females of Oc. *triseriatus* that had engorged 6 days before mating with LACV-infected males, by which time their oocytes were already surrounded by eggshell, did not lay infected eggs in that (first) ovarian cycle. Clearly, transovum infection (invasion of ova through the micropyle during ovulation) had not occurred. In the second ovarian cycle, a maternal infection rate of 0.4 was recorded and a filial infection rate of 0.64 (Thompson, 1979). In experiments in which females were blood fed either 6–8 h before or up to 7 days after mating with vertically infected males, no infected eggs were laid in the first ovarian cycle, but transmission rates of 28%, 61% and 56% were recorded for venereally infected females in their second, third and fourth ovarian cycles, respectively (Patrican and DeFoliart, 1987). Citing their observation that 81% of the eggs laid by wild Oc. *triseriatus* are from the first ovarian cycle, Patrican and DeFoliart (1987) commented that even high rates of infection in egg batches from the second and third ovarian cycles of venereally infected females would make no more than a modest contribution to offsetting the erosion of virus prevalence during vertical transmission (cf. Section 44.6.5).

A proportion of a batch of female Oc. *triseriatus* that had become infected with LACV during forced matings, and which developed disseminated infections, could infect suckling mice by bite (Thompson and Beaty, 1978). When 204 venereally infected females were placed with suckling mice, seven transmitted LACV on day 12 post-mating, and 13 transmitted the virus on day 22 post-mating. On day 32 post-mating, ten out of 44 transmitted the virus (Patrican and Defoliart, 1987).

Feeding by uninfected mosquitoes on hosts circulating LACV-specific neutralizing antibodies

could later affect venereal transmission to those females. Two batches of Oc. *triseriatus* fed on chipmunks that were either with or without such antibodies, and 5–11 days later the mosquitoes were caged with vertically infected males. Of the females that imbibed neutralizing antibodies, only 24% later transmitted virus to suckling mice, compared with 38% of females that had fed on chipmunks without neutralizing antibodies ($p \approx 0.001$) (Thompson, 1983a).

44.5.3 *Simulation model*

Whether venereal transmission contributes significantly to the perpetuation of an arbovirus is difficult to test in the field, but modelling can provide a perspective. The model described below involves a population of newly emerged adults of which a proportion are vertically infected, and in which virus is transmitted venereally from vertically infected males to uninfected females. It calculates the contribution of venereal transmission, during two gonotrophic cycles, to the perpetuation of the virus by females that are infected venereally, and only venereally, and that lay infected eggs. It can also be used to quantify horizontal transmission of the virus through the bites of venereally infected females. Any increase in infection rate among emerging adults increases the proportion of the male population capable of venereally infecting females, but decreases the proportion of the female population that can become venereally and only venereally infected. Females that are already vertically infected at emergence and that mate with vertically infected males are excluded from the proportion of the female population that is infected venereally and only venereally.

The model covers two gonotrophic cycles because experimental studies showed a delay between the time of venereal infection of females and their ability to transmit horizontally by bite or vertically to their offspring. The model entails the following assumptions: (i) at emergence the sex ratio is 1:1; (ii) infected males do not have a mating advantage or disadvantage; (iii) all females mate only once, before the start of the first gonotrophic cycle, when some females become venereally infected; (iv) the duration of the first and second gonotrophic cycles is the same; (v) the second gonotrophic cycle starts on the day following the end of the first gonotrophic cycle; (vi) the daily survival rate is independent of age and of infection; (vii) vertically infected females transmit virus by bite at the start of the first gonotrophic cycle and lay vertically infected eggs at the end of the first gonotrophic cycle; (viii) venereally infected females do not transmit virus by bite until the start of the second gonotrophic cycle, and do not lay infected eggs until the end of the second gonotrophic cycle.

The proportion of the female population that is venereally and only venereally infected and that is capable of laying vertically infected eggs at the end of the second gonotrophic cycle (A) is given by

$$A = \left[(1-a)\,(b\,c)\,p^{2n+3}\right] \tag{44.7}$$

where a and b are the proportions of the adult female population and the adult male population, respectively, that are vertically infected, c is the efficiency of venereal transmission, p is the daily survival rate, and n is the duration of the gonotrophic cycle in days.

The proportion of the female population that is vertically infected and that survives to lay vertically infected eggs at the end of the first gonotrophic cycle, plus the proportion that survives to lay vertically infected eggs at the end of the second gonotrophic cycle (B), is given by

$$B = (a\,p^{n+3}) + (a\,p^{2n+3}) \tag{44.8}$$

The results of a specimen run of the model are shown in Table 44.5, where the values attributed to the different variables are also detailed. The range of infection rates at emergence covers three orders of magnitude; the lower rates correspond to minimum infection rates recorded in the field for Oc. *triseriatus* infected with La Crosse virus; the extended range reveals how the rates of venereal transmission and vertical transmission are affected differently by increases in the proportion of the population that is vertically infected at emergence.

Table 44.5 Estimates from a simulation model (Eqns 44.7 and 44.8) of the proportions of an adult female population that: (i) being vertically infected at emergence, survive to oviposit at the ends of the first and second gonotrophic cycles, or (ii) become venereally and only venereally infected and survive to oviposit at the end of the second gonotrophic cycle. The population has the following characteristics: adult female and male vertical infection rates are identical ($a = b$); c, efficiency of venereal transmission, 0.75; period before first gonotrophic cycle, 3 days; n, length of each gonotrophic cycle, = 6 days; p, female daily survival rate, 0.9. With increase in $a = b$, B (see note) increases progressively, whereas A (also see note) reaches a maximum at $a=b=0.5$ and then decreases at the same rate. The value A/B decreases as $a = b$ increases. The four lowest levels of vertical transmission rate at emergence (0.002–0.02) have been recorded from natural populations of *Oc. triseriatus* infected with La Crosse virus.

$a = b$	A	B	A/B
0.002	0.00031	0.00118	0.263
0.005 *	0.00077	0.00296	0.260
0.008	0.00122	0.00474	0.257
0.02	0.0030	0.0118	0.252
0.05 **	0.0073	0.0296	0.247
0.08	0.0114	0.0474	0.240
0.2	0.0247	0.1186	0.208
0.5 ***	0.0386	0.2965	0.130
0.8	0.0247	0.4744	0.052

$a = b$: proportion of the adult female and male populations that is vertically infected at emergence. *, ≡ MIR 5/1000. **, ≡ MIR 50/1000. ***, ≡ MIR 500/1000.
A: proportion of the adult female population that becomes venereally and only venereally infected and that survives to oviposit at the end of the second gonotrophic cycle (Eqn 44.7).
B: sum of the proportions of the adult female population that are vertically infected and that survive to oviposit at the end of the first and end of the second gonotrophic cycles (Eqn 44.8).
A/B: proportionate increase to perpetuation of virus by vertical transmission through two gonotrophic cycles that is due to venereal transmission.
MIR = Minimum infection rate.

The column A/B shows the proportionate increase to the perpetuation of virus by vertical transmission through two gonotrophic cycles due to venereal transmission. When vertical infection rates at emergence are 0.002 to 0.08, this increase is within the range 24–26%. The rate of venereal transmission is maximum when the vertical transmission rate at emergence (the maternal infection rate) is 0.5 (column A). As the vertical infection rate rises above 0.5, the venereal transmission rate progressively declines (Table 44.5).

The equations can be used also to estimate the contribution to horizontal transmission, and potentially to virus amplification, made by venereally infected females that survive to transmit virus by bite at the start of the second gonotrophic cycle. A specimen run of the model (not detailed) showed this to be almost twice as great as the contribution to vertical transmission made by venereally infected females ovipositing at the end of the second gonotrophic cycle.

Should the efficiency of venereal transmission be substantially lower than the 0.75 used as a guide in this run, the contribution of venereal transmission to perpetuation of the virus, and to its amplification, would be much lower. If with flaviviruses there is transovum transmission, some venereally infected females might transmit virus vertically to their progeny from the first gonotrophic cycle.

44.5.4 Conclusions

In the absence of any knowledge of venereal transmission in natural populations of mosquitoes, views as to whether venereal transmission might enhance the perpetuation of arboviruses can only be speculative. Males capable of transmitting an arbovirus during coition must themselves have been infected vertically. Further, the contribution of venereal transmission to the infection of previously uninfected females varies with the proportion of adult females infected at emergence in that population. At most, it can be postulated that venereal transmission can increase the contribution of previously uninfected females in a population to the horizontal transmission of an arbovirus to amplifying hosts, and increase vertical transmission of the arbovirus to the following filial generation. It is difficult to identify selective forces that might act to increase rates of venereal transmission.

44.6 VERTICAL TRANSMISSION

Vertical transmission is the transference of an infectious agent from a male or female parent to its progeny. Basic and theoretical aspects of vertical transmission discussed in Sections 41.2.3 and 41.4 provide information that may be necessary for an understanding of this section. Where vertical transmission plays a role in the transmission cycles of mosquito-borne arboviruses it is never as the sole modality, but is always in association with horizontal transmission. Vertical transmission can readily be investigated in the laboratory, at least for arboviruses that can be propagated in colonized mosquitoes. In nature, vertical-transmission rates are often low or very low, so it can be difficult to demonstrate in wild mosquito populations, but the presence of arbovirus in the developmental stages or in adult male mosquitoes provides clear evidence of vertical transmission.

Vertical transmission of arboviruses has been demonstrated in wild populations of a number of mosquito species, but in most cases its importance for perpetuation of the virus is not known. Simulation models described in Section 41.4.2 identify variables that are involved in the integration of vertical transmission with horizontal transmission in the perpetuation of an arbovirus. Measurements of those variables are lacking for most mosquito-borne viruses, but field data for three bunyaviruses show the importance of vertical transmission to a greater or lesser degree (Section 44.6.2.a).

44.6.1 Laboratory studies

The results of laboratory experiments on vertical transmission should be interpreted cautiously. Many such experiments, especially with flaviviruses, start with intrathoracic inoculation of an arbovirus into adult female mosquitoes, to be followed some days later with ingestion of an uninfected blood meal. This procedure bypasses both the midgut-infection and midgut-escape barriers and rapidly leads to a disseminated infection. As noted later, vertical transmission to the progeny of orally infected females generally does not occur in the gonotrophic cycle in which the females become infected, but in the next and any subsequent gonotrophic cycles. Consequently, the effective vertical-transmission rates for a host-virus combination may be higher in the laboratory than in the field, where females must survive natural hazards to complete a second gonotrophic cycle.

(a) Experimental studies with bunyaviruses

Laboratory studies have involved strains of the California encephalitis virus group and species of *Ochlerotatus* as hosts. Whether stable infections develop in any of these virus/host associations is discussed in Section 44.6.5.

When females of *Oc. triseriatus* were orally infected with La Crosse virus, none of their F_1 progeny were infected, but progeny from the second and third gonotrophic cycles were infected, the vertical-transmission rate being higher in the third cycle. LACV could not be recovered from ovarian tissues during the first 6 days after an infected blood meal, but was detected on the seventh day. It appears that, in the first gonotrophic cycle of orally infected females, virus does not reach and invade the oocytes while they are still unprotected by chorion. In contrast, F_1 progeny from intrathoracically inoculated *Oc. dorsalis* and *Ochlerotatus melanimon* were infected (Miller *et al.*, 1979; Turell *et al.*, 1982a,d).

When females of *Oc. melanimon* that had been intrathoracically inoculated with CEV subsequently underwent three ovarian cycles, the vertical-transmission rate to the progeny from the first ovarian cycle (0.21) was higher than that to the second (0.14) or third (0.13) (Turell *et al.*, 1982a). A similar experiment involving a line of *Oc. dorsalis* that had been selected for vertical transmission of CEV gave vertical-transmission rates for three successive ovarian cycles of 0.91, 0.90 and 0.51 (Turell *et al.*, 1982d).

California encephalitis virus was recovered from vertically infected *Oc. melanimon* eggs that had been stored for up to 19 months. Neither time

since oviposition (3–19 months), nor storage temperature (4°C or 27°C) or repeated freezing and thawing affected infection rates in F_1 adult females. Vertical infection with CEV in *Oc. dorsalis* did not affect the survival of embryos, larvae or adults. However, vertically infected larvae of both *Oc. melanimon* and *Oc. dorsalis* took longer to develop to the pupal stage than did their uninfected siblings. If that situation obtained in the field it might reduce fitness by prolonging the risk of predation. With both species, similar numbers of male and female adults were infected (Turell *et al.*, 1982c). After vertical transmission of CEV through successive generations of *Oc. dorsalis*, infected F_5 females that fed on infant mice transmitted the virus (Turell *et al.*, 1982d).

A study was undertaken on the effects of vertical infection of *Oc. triseriatus* with LACV on a number of biological variables, particularly the effects in large and small mosquitoes (Patrican and DeFoliart, 1985). Eggs collected in the field were reared to provide a parental generation, some of the adults being inoculated with LACV. Their vertically infected and uninfected (control) progeny were reared through five filial generations (F_1 to F_5). By regulating larval nutrition in each generation, the adults that developed were either small or large. Probably, the small adults were closer in size to most wild *Oc. triseriatus*. The mean duration of the larval stage was shorter for the well-fed larvae (14–16 days) than for the poorly fed (25–33 days) ($p < 0.01$), and in an assessment of all tests larval survival was 6% greater for large than small larvae.

No significant differences for a number of characteristics were found between batches of *Oc. triseriatus* that were either uninfected or vertically infected with LACV – hatching success, duration of larval stage, sex ratio, time from blood feeding to ovarian maturation, fecundity, or survival – whether the larvae were reared under conditions of adequate nutrition or of nutritional stress. However, the mean rate of vertical transmission in four generations, as measured in the adult stage, was distinctly higher in small mosquitoes than in large, for both females and males ($p = 0.001$) (Table 44.6A). Also,

Table 44.6 Effects of the body size of *Ochlerotatus triseriatus* on (A) the mean rate of vertical transmission of La Crosse virus through four generations, and (B) the mean rate of transmission of LACV from vertically infected females to suckling mice during their first and second blood meals. (After Patrican and DeFoliart, 1985.)

A. Mean rate of vertical infection

	Females		*Males*	
Class sampled	*Rate (%)*	*n*	*Rate (%)*	*n*
Large	60.7	333	55.4	312
Small	73.0	224	69.3	310

B. Mean rate of oral transmission

	First blood meal		*Second blood meal*	
Class sampled	*Rate (%)*	*n*	*Rate (%)*	*n*
Large	52.3	331	54.2	275
Small	67.7	307	67.5	268

Eggs of *Oc. triseriatus* from a site of enzootic LACV transmission were used to establish a parental generation. Some parental females were inoculated with LACV (isolated from *Oc. triseriatus* larvae and used after one mouse-brain passage). Two groups of large mosquitoes were established, with either vertically infected or uninfected eggs of the F_1 generation; two comparable groups were set up with small mosquitoes, with eggs of the F_2 generation. Mean vertical transmission rates were determined for generations F_1–F_4 of large mosquitoes, and generations F_2–F_5 of small mosquitoes.

the ability of vertically infected adult females to transmit LACV to suckling mice was significantly greater in small females than in large at both the first blood meal and the second (p = 0.001) (Table 44.6B).

(b) Experimental studies with flaviviruses

When DENV, JEV or SLEV were intrathoracically inoculated into gravid females of *St. albopicta*, i.e. after the oocytes had been surrounded by eggshell, the progeny of some of the females became infected. For example, mated females of *St. albopicta* were blood fed and, 5 days after the completion of ovarian development, were inoculated with JEV. No eggs laid during days 1–3 post-inoculation produced infected progeny, but a small proportion of eggs laid 3–4 days after inoculation, and a greater proportion laid 4–5 days after inoculation, produced infected progeny. This timing indicated that, in the females that became infected with JEV, the developing oocytes had been surrounded with chorion before virions could invade them (Rosen, 1987b, 1988).

An early idea of the means of vertical transmission of flaviviruses proposed that virions adhered to the eggshells as ova passed through the female genital ducts during ovulation, and that newly hatched larvae ingested some virions and were orally infected. There is no evidence that supports this idea, and there is evidence that contradicts it. *Stegomyia albopicta* larvae infected with JEV had hatched from eggs that had been dried and kept at room temperature for up to 2 months. Further, when uninfected *St. albopicta* eggs had hatched in a medium containing a high density of JEV, and the larvae had remained there for 21 h, only one larva in 64 became infected (Rosen *et al.*, 1978). In other experiments, females of *St. aegypti* were intrathoracically inoculated with YFV, and 5 days later were fed on mice. Of the eggs laid by these females, one half were surface sterilized with sodium hypochlorite solution before hatching, while the remainder hatched untreated. All were then allowed to develop to F_1 adults. YFV was detected in 42 of 227 pools of the adult F_1 progeny (n = 28,530). Of the 42 positive pools, 26 were from surface-sterilized eggs and 16 from non-sterilized eggs (Aitken *et al.*, 1979).

That venereal transmission of virus, from males to females, can lead to vertical transmission was shown experimentally. Male *St. albopicta* were parenterally infected with DENV-1, and virgin females were given a non-infective blood meal. The mosquitoes mated 14 days after the males had been infected and 3 days after the females had taken their blood meals, at which time their oocytes were mature and enclosed in an impermeable chorion. No eggs laid during the period 0–72 h post-mating produced infected larva (Table 44.7). The first infected eggs were laid 73–96 h after mating, i.e. after an interval long enough for viral replication in the female reproductive tract, and thereafter the minimum infection rates increased (Rosen, 1987a).

From his experimental findings, Rosen (1987b, 1988) postulated that, when female mosquitoes have been venereally infected with a flavivirus, virions that have accumulated in the reproductive tract invade some oocytes through their micropyles at the time of ovulation, just before oviposition. He further postulated that that is the mechanism of vertical infection by flaviviruses generally. In contrast to transovarian transmission, such transovum transmission permits the infection of progeny after a single maternal blood meal, especially if oviposition is delayed. Theoretically, transovarian transmission of flaviviruses should be possible in infected females that survive to complete further gonotrophic cycles.

Invasion through micropyles is the only feasible mechanism for the infection of mature oocytes by flaviviruses, but it is supported only by circumstantial evidence and is difficult to envisage. Mature oocytes are surrounded by an impermeable chorion, which will become the outer layer of the eggshell. Near the anterior pole of the oocyte, the chorion is structurally modified to form a micropyle, a structure that is open to permit the entry of sperm as oocytes leave the ovary and pass along the oviducts – the process of ovulation (Volume 1, Section 3.1). The passage of oocytes and the

Table 44.7 Vertical transmission of dengue virus 1 from venereally infected females of *Stegomyia albopicta* to their offspring. Minimum infection rates in the larvae (a measure of the vertical transmission rate) are shown in relation to time elapsed between mating (when the females became infected) and oviposition. (From Rosen, 1987a.)

Mating to oviposition (h)	*No. of larvae tested*	*Number of pools*		*MIR*
		Positive	*Tested*	
0–24	86	0	11	–
25–48	2456	0	39	–
49–72	7756	0	80	–
73–96	6968	1	74	0.14
97–120	3129	4	33	1.28
121–144	1208	4	16	3.31
145–264	850	4	38	4.70

In the first stage of the experiment, males of *St. albopicta* were intrathoracically inoculated with DENV-1, and virgin females were given a non-infectious blood meal. These mosquitoes later mated – 14 days after the males had been infected and 3 days after the females had taken their blood meals. Eggs were collected daily, held during embryonic development, and then stimulated to hatch. The F_1 larvae were tested for infection with DENV-1. Eventual analysis of a sample of the parental females showed 27% to have been venereally infected.

probable contact of the micropyle with the orifice of the spermathecal duct during ovulation are best known for *Anopheles* (Volume 2, Section 40.7.4). The delay that is found between venereal infection of gravid females and first infection of oocytes suggests that virus cannot enter the oocytes with sperm. Rather, replication of virus within the oviducts is needed to permit entry through the micropyle as the oocytes move through the oviducts.

The vertical transmission of DENV-1 from parenterally infected females of *St. albopicta* to their F_1 progeny was low, but that from vertically infected F_1 females to their progeny was higher. Thus, in one study that started with parenterally infected females, the vertical-transmission rates were: $P \rightarrow F_1$, 0.004; $F_1 \rightarrow F_2$, 0.029; $F_2 \rightarrow F_3$, 0.028. Shroyer (1990) concluded that in vertically infected females the virus might be able to invade germ cells within the ovary, permitting trans-ovarian transmission. A similar trend was shown in *St. aegypti* when females of the parental generation were inoculated with DENV-3; the vertical-transmission rates were: $P \rightarrow F_1$, 0.028; $F_2 \rightarrow F_7$, 0.086 to 0.126 (Joshi *et al.*, 2002).

In most studies of vertical transmission of JEV in *Culex tritaeniorhynchus*, the vertical-transmission rate was about four times higher when measured in fourth-instar F_1 larvae than in F_1 adults. The reason was not known (Rosen *et al.*, 1989).

Vertical transmission of WNV in *Cx. pipiens* shows temperature sensitivity. In one briefly described study, 1618 F_1 progeny from parental females that had been inoculated with WNV were tested for WNV. The virus was not detected in any of 641 progeny reared at 18°C, or in 933 progeny reared at 26°C, but it was recovered from one pool of seven larvae from among 44 reared at 26°C (Turell *et al.*, 2001). Female *Cx. pipiens* were intrathoracically inoculated with WNV, kept for 7 days at 26°C and then blood fed. Their F_1 adult progeny, grouped in pools of ≥25 males or females, were tested for WNV. The minimum filial infection rate of male + female F_1 adults that had been kept at 18°C was ≈1.4/1000; of those kept at 26°C it was 2.1/1000 (Dohm *et al.*, 2002b).

In summary, some flaviviruses can be transmitted vertically even when the oocytes are mature and surrounded by chorion before dissemination of the virus. The probable mechanism is entry of virions through the oocyte micropyles during ovulation (so-called transovum transmission), but how this occurs is not clear. During the second

and later gonotrophic cycles, invasion of oocytes by residual virus may be possible. Once vertical infection has occurred, by whatever means, transovarian transmission to the following generation should be possible.

(c) Experimental studies with alphaviruses

After females of *Culiseta melanura* had fed on chicks viraemic with EEEV, fluorescent-antibody staining showed virus to be present in most organs, including the lateral oviducts (n = 40) and common oviduct (n = 29) but not the ovarioles (n = 90). The presence of EEEV in pools of egg rafts laid on days 15 and 16 post-infection was ascribed to transovum transmission (Scott *et al.*, 1984).

Females of *Oc. vigilax* were inoculated with Ross River virus (RRV), blood fed 5 days later, and induced to oviposit 3 to 5 days after feeding. The eggs laid were washed in sodium hypochlorite solution, dried, stored for 55 days, and then flooded with diluted seawater. Of 122 adult F_1 progeny, two were infected. Four days after oral infection with RRV, virus was present in the ovaries of 50% of females, but the location of virus within the ovaries was not determined. On this evidence, Kay (1982) tentatively suggested that vertical transmission is a potential mode of survival for RRV.

44.6.2 Vertical transmission in wild mosquito populations

Assertions of the epidemiological importance of vertical transmission made by some investigators, following discoveries in nature, have not been universally accepted. Fine and Sylvester (1978) commented that reports of infectious agents being transmitted vertically are not, in themselves, epidemiologically significant statements. Rosen (1981) considered that the epidemiological significance depends on a number of independent variables, e.g. relative size of the vector and host populations, and frequency of blood feeding by the vector. The basic problem is that infectious agents can be perpetuated without loss by vertical transmission alone only when certain conditions are met and so, for the perpetuation of most and possibly all mosquito-borne viruses, vertical transmission must be supplemented by virus amplification during intervening cycles of horizontal transmission (Section 41.4.2).

A situation in which vertical transmission of mosquito-borne viruses can be epidemiologically important is when a virus must survive through periods of adverse climatic conditions. There are known examples of persistence within dormant, desiccation-resistant aedine eggs being important for perpetuation of viruses (Section 44.7.3). Persistence of virus within diapausing adult females has been discounted on physiological grounds, but the use of new virological techniques of higher sensitivity has produced evidence that certain viruses may possibly survive through winters in vertically infected diapausing females (Section 44.7.4.b).

Despite an extensive literature on the vertical transmission of arboviruses in mosquitoes, its epidemiological significance remains uncertain for most known examples, and this can be ascribed in part to the general failure to develop simulation models. Fine's (1975) 'fundamental vertical transmission equation' (Section 41.4.2.c), which concerns maintenance by vertical transmission alone, is too complex to provide a practical example. The model of combined vertical and horizontal transmission of Keystone virus formulated by Fine and LeDuc (1978) (Section 41.4.2.b), although not validated, provides a useful guide for cases in which infections in the mosquito host are non-stable.

(a) Bunyaviridae

Vertical transmission in the field has been recorded for certain strains of California encephalitis virus (CEV) in species of *Ochlerotatus*. Because it is one of the very few cases subjected to modelling, we examine the transmission of CEV in populations of *Oc. melanimon* in the Sacramento Valley, California. On slender, circumstantial evidence, it was postulated that CEV persists in

these populations largely through vertical transmission. A potential for vertical transmission was demonstrated in females intrathoracically inoculated with CEV, which produced filial infection rates of 0.03–0.54 in the progeny of individual F_1 females. Vertical transmission to another generation could not be examined because the mosquitoes would not mate under laboratory conditions. During the years 1970–1980, screening *Oc. melanimon* collected in the Sacramento Valley showed CEV (of unspecified strain) to be present in each of the months May to October with mean monthly MIRs of 0.7–0.81 (Table 44.8). Referring to Fine and LeDuc's (1978) simplified model of transmission (Section 41.4.2.b), Turell *et al.* (1982d) argued that, assuming an initial prevalence rate of 0.5/1000 and a vertical transmission rate of 0.2 (representative of the measured values cited above), the CEV would disappear within a very few generations unless amplified during horizontal transmission. However, if the initial prevalence rate of 0.5/1000 represented stable infections, the prevalence rate in each succeeding generation would remain at approximately that level, because these mosquitoes would transmit virus to nearly all their progeny. If only some infections were stable, the prevalence of infection at the start of any generation would reflect that proportion. On the available evidence, the means of perpetuation of CEV in these populations is unclear. The prevalence of infection through the season shows no accumulative increase, such as might be expected from amplification during horizontal transmission (Table 44.8). However, there is no firm evidence of stable infections.

Table 44.8 Isolations of California encephalitis virus (strain not specified) from *Ochlerotatus melanimon* collected in the Sacramento Valley, California, from 1970 through 1980, by month of isolation. (From Turell *et al.*, 1982d.)

Month of capture	*No. of isolates*	*Number tested*	*Mean MIR * (no./1000)*
May	3	11,094	0.27
June	20	24,597	0.81
July	9	17,863	0.50
August	9	15,225	0.59
September	4	23,016	0.17
October	4	14,670	0.27
Total	49	106,495	0.46

* MIR, minimum infection rate.
The virus isolated from *Oc. melanimon* in the Sacramento Valley was similar to, or identical with, the prototype strain of California encephalitis virus. (LeDuc, 1979; citing a personal communication from W.C. Reeves.)

The best substantiated cases of perpetuation of mosquito-borne viruses through the coupling of vertical transmission and horizontal transmission with amplification concern three bunyaviruses, and are described elsewhere in this volume: (i) transmission of Keystone virus in *Ochlerotatus atlanticus*, analysed with simulation models (Section 41.4.2); (ii) transmission of La Crosse virus in *Oc. atlanticus* (Section 45.5.6.d); and (iii) transmission of Rift Valley fever virus in eastern and southern Africa (Section 45.6.4.c).

(b) Flaviviridae

Vertical transmission in natural populations of their hosts has been reported for at least four flaviviruses. Claims that certain flaviviruses survive through periods of climatically adverse conditions by means of vertical transmission may be correct, but none has been supported with supplementary evidence or tested with simulation models.

Dengue virus DENV-2 was isolated from wild-caught male *Diceromyia taylori* and *Diceromyia furcifer* in Côte d'Ivoire (Roche *et al.*, 1983) and Senegal (Saluzzo *et al.*, 1986; Diallo *et al.*, 2003). In Jalore, India, DENV-3 was isolated from adult females reared from wild-caught larvae of *St. aegypti*. It was detected in each of the months of January to April 1993, during a dengue outbreak, when the MIRs ranged from 45/1000 to 142/1000 (mean 98.2; $n = 224$). Only females were infected. No DENV was recovered during the period May to December 1993 ($n = 164$) (Joshi *et al.*, 1996). In Singapore, dengue virus was detected in wild-caught males of *St. aegypti* (MIR = 13.3) and *St. albopicta* (MIR = 21.5) (Chung *et al.*, 2001). In Rangoon, Myanmar, the following MIRs were determined for *St. aegypti*: wild-caught larvae, 0.48;

adult males reared from wild-caught larvae, 0.26; adult females, 0 (Khin and Than, 1983). From ten locations in Trinidad where cases of dengue fever had occurred, eggs and larvae of *St. aegypti* were collected and reared to adults. When tested for DENV, the single pool from one location was infected (MIR = 0.54); for the combined collections the MIR was 0.09 (Hull *et al.*, 1984).

Yellow fever virus. Three isolates of YFV were obtained from 44 pools of wild Furcifer Group (*Diceromyia furcifer* and *Di. taylori*) males in eastern Senegal (MIR = 2.37; *n* = 1266). The investigators suggested that vertical transmission might be important for maintenance of the virus, enabling it to survive dry seasons within drought-resistant aedine eggs. However, they were puzzled by the fact that, whereas eclosion of Furcifer Group adults ended in September, YFV had not been isolated from the adult males until November and December. In the insectary, they had observed male *St. aegypti* imbibing blood that had been discharged by females, but they had no knowledge of this occurring in nature or in other species. Cornet *et al.* (1979b) concluded that their isolation of YFV from adult male Furcifer Group mosquitoes was of uncertain significance. Nevertheless, their finding was cited by a number of authors as evidence that vertical transmission plays an important role in the maintenance of YFV in sylvatic cycles in West Africa (Germain *et al.*, 1981; Salaun *et al.*, 1981; Digoutte *et al.*, 1995).

An investigation into vertical transmission was undertaken during a yellow fever outbreak in the Koungheul area of Senegal, where villages were located in 'scrubby savannah' and water was stored both indoors and outdoors for domestic uses. The scale colour of the *St. aegypti* population was consistent with that of peridomestic *St. aegypti formosus*. For newly emerged adult *St. aegypti* reared from wild-caught larvae the MIRs were: males, 5.3/1000 (*n* = 189); females, 12.1/1000 (*n* = 165). Remarkably high MIRs were estimated for mosquitoes captured at human bait: *St. aegypti*, males 121.2 (*n* = 33); females 54.5 (*n* = 312); *Di. furcifer*, females 105.3 (*n* = 10). The infected males of *St. aegypti* caught at bait must have been vertically infected, but that could not be said with certainty of the females. Fontenille *et al.* (1997) suggested that in the Koungheul area YFV might persist between rainy seasons in vertically infected eggs laid in peridomestic sites that dry up, such as used tyres and pots.

During an epizootic of YF among howler monkeys in forested areas of Trinidad during December 1988 and January 1989, YFV was isolated from *Haemagogus janthinomys* and *Sabethes chloropterus* (Rawlins *et al.*, 1990). During January 1989, in an attempt to demonstrate vertical transmission of YFV, aedine eggs were collected in ovitraps placed in parts of the island where sylvatic yellow fever had occurred during the previous 2 months. Of the 52,632 eggs that were harvested, hatched, reared and screened for YFV, 87% were of *Haemagogus* and 12% of *Ochlerotatus* (*Protomacleaya sensu auctorum*). No isolations of YFV were made (Rawlins *et al.*, 1991). Success was achieved in eastern Amazonia in 1998 when, a month after an epizootic of YF which killed many howler monkeys, YFV was isolated from pools of nulliparous females of *Hg. janthinomys* (MIR = 24.4; MLE = 29.5) (Mondet *et al.*, 2002).

Japanese encephalitis virus. How JEV overwinters in the cool-temperate region has been the subject of speculation and research in Japan, Korea and China. In Japan, in years of active transmission, very large numbers of female *Cx. tritaeniorhynchus* that appeared in early spring were screened but no isolations of JEV were made. For example, JEV was absent from over 90,000 adult females in the Nagasaki area collected between March and late May through a 10-year period (Table 45.17).

A study of vertical transmission in a Taiwanese population of *Cx. tritaeniorhynchus* was undertaken during a period of 3.5 years. Screening almost 400,000 wild-caught, fourth-instar larvae led to a single isolation of JEV, whereas screening 140,000 wild-caught adult females resulted in 163 isolations. In the laboratory, the vertical transmission rate among the progeny of infected females was 0.001–0.01. The seemingly low infection rate among the wild larvae was consistent with that among the wild adult females if the vertical transmission rate was

taken into consideration. Given an adult female infection rate of 163/140,000, a vertical transmission rate of 0.01 should yield about five isolations from 400,000 larvae, while a vertical transmission rate of 0.001 should yield about one isolation from 800,000 larvae (Rosen, 1987c).

Evidence of vertical infection with JEV has been obtained from wild populations of *Cx. pipiens pallens* in China (Huang, 1982, review), and of *Cx. tritaeniorhynchus*, *Culex vishnui* and *Mansonia indiana* in India (Dhanda *et al.*, 1989; Arunachalam *et al.*, 2002). At locations in South India where Japanese encephalitis was endemic and *Cx. tritaeniorhynchus* was the main vector, wild-caught immatures were reared and tested as adults. JEV was isolated from pools of *Cx. tritaeniorhynchus* (MIR = 0.017) and from a single pool of *Cx. fuscocephala* (MIR = 1.05) (Thenmozhi *et al.*, 2001).

West Nile virus was identified in a pool of four adult male mosquitoes, identified as *Culex univittatus* group, at a point along the Kenya–Uganda border (Miller *et al.*, 2000). It was also isolated from overwintering larvae of *Culex erythrothorax* in Utah (Phillips and Christensen, 2006). Evidence of a possible role of vertical transmission in the overwintering of WNV in North America, based on its isolation from hibernating female *Cx. pipiens*, is presented in Section 44.7.4.

(c) Togaviridae

In an examination of **Eastern equine encephalitis virus** distribution in orally infected females of a long-established laboratory colony of *Cs. melanura* by the fluorescent antibody technique, the virus was detected in the common and lateral oviducts but not in the ovarioles. However, EEEV was isolated from a proportion of egg rafts (Scott *et al.*, 1984).

Field studies have been undertaken on populations of *Cs. melanura* at localities at which Eastern equine encephalitis was endemic. In a swamp near the southern coast of Alabama, EEEV was found to be infecting birds and mosquitoes. Here, *Cs. melanura*, which was thought to be the enzootic vector, overwintered in the larval stage. To test for possible vertical transmission of EEEV, third- and fourth-instar larvae of *Cs. melanura* (n = 2205) were collected in February 1961 from an area that had yielded most isolations of EEEV in the previous November, but no evidence of infection was obtained (Sudia *et al.*, 1968). At Pocomoke Swamp, Maryland, no isolations of EEEV were made from 2503 *Cs. melanura* larvae (Sprance, 1981). At Toad Harbor Swamp, New York State, seven isolates of EEEV were obtained from apparently non-blood-fed females in 1976 (n = 2656) and 12 isolates in 1977 (n = 3977); however, high parity rates among those females made the evidence equivocal, and the results were interpreted as negative (Morris and Srihongse, 1978). During a screening programme carried out in central New York State during the period 1983–92, 119 isolations of EEEV were made from adult *Cs. melanura* (n = 284,586) and 14 isolations were made from *Cs. morsitans* (n = 97,208); both species are enzootic vectors. In addition, 20 isolations were made from species regarded as epizootic vectors (n = 653, 382), all during seasons of enzootic or epizootic activity. No isolations were made outside those seasons, so no evidence of vertical transmission was obtained (Howard *et al.*, 1994).

Third- and fourth-instar larvae of *Ochlerotatus camptorhynchus* collected in early autumn from salt marsh in Victoria, Australia, were reared to the adult stage, sorted by sex, and grouped into pools for virus isolation (n = 1006). Two viruses were isolated from pools of female mosquitoes, namely **Sindbis virus** (MIR = 2.0) and **Ross River virus** (MIR = 1.0) (Dhileepan *et al.*, 1996). Isolates of Ross River virus were obtained from a pool of six male *Oc. vigilax* and from a single male of *Macleaya tremula* caught by trapping in Western Australia (Lindsay *et al.*, 1993).

44.6.3 Inheritance

Experiments with colonized mosquitoes have shown that, by means of selection, lines can be developed that have an increased or a decreased vertical-transmission rate for a particular infectious agent. An early attempt at selection involved a

colony of *Stegomyia albopicta* and San Angelo virus (a strain of California encephalitis virus), not a natural combination. Initially, 46 females were inoculated intrathoracically with the virus and later blood fed, inducing oogenesis. Based on their filial infection rates, the F_1 progeny fell into three groups of seven, 34 or five females: (i) progeny of seven females – none infected; (ii) progeny of 34 females – some progeny of all 34 were infected, and infection rates were 0.9–51.3%; (iii) progeny of five females – some progeny of all five were infected, and infection rates were 83.3–94.6%. The progeny of one of the group of five females were subjected to selection, and in each later generation females for breeding were selected from families with a high filial infection rate. This resulted in filial infection rates of 1.0 in the last four of seven generations (Tesh and Shroyer, 1980). Clearly, some aspect of vertical transmission was under genetic control.

Studies were undertaken into the inheritance of factors controlling vertical transmission of La Crosse virus in Oc. *triseriatus*. In the first, lines were developed by selection from two laboratory-adapted strains of Oc. *triseriatus*: the Holmen strain which was refractory to vertical transmission; and the AIDL strain which was permissive. In each generation, breeding females were selected from 'Holmen line' families with a low filial infection rate, and from 'AIDL line' families with a high filial infection rate. Over two generations, the effect of selection on the Holmen line was a marked fall in both the transovarian infection rate (the proportion of females passing the virus to their progeny) and the filial infection rate (the proportion of the progeny of an infected female that is infected through vertical transmission). Over three generations, the effect of selection on the AIDL line was a discernible increase in the transovarian infection rate but no clear change in the filial infection rate. When reciprocal crosses were made between progeny from Holmen F_2 females and progeny from AIDL F_3 females, the adult female progeny from the reciprocal crosses had transovarian infection rates not significantly different from those of the permissive (AIDL) strain, but significantly different from those of the refractory (Holmen) strain (Graham *et al.*, 1999). A later study with the same Holmen and AIDL strains suggested that at least three loci condition permissiveness for transovarian transmission. A single quantitative trait locus was found on chromosome-2, while chromosome-3 appeared to have two quantitative trait loci (QTLS) which did not segregate independently. Alleles at the three loci contributed additively, but alleles at the chromosome-2 QTL had stronger effects than those at the chromosome-3 QTLs (Graham *et al.*, 2003).

In a number of early investigations it was found, with both bunyaviruses and flaviviruses, that strains of a particular virus that were of different geographical origins varied in efficiency of vertical transmission in a particular mosquito host (e.g. Tesh, 1980; Miller *et al.*, 1982). Measurements of transmission dynamics are realistic only when host and parasite strains have been collected recently and from locations in which they occur sympatrically. The selective forces acting on hosts and parasites are likely to differ in different locations and at different times.

44.6.4 Stable and non-stable infections

The characteristics of stable and non-stable infections are described in Section 41.3.1. Essentially, stable infections ensure perpetuation of a virus through successive generations of the host by vertical transmission. Because the virus infects the primary germ cells during embryogenesis all female gametes are infected, and the virus is transmitted to the following generation without any reduction in prevalence rate. In non-stable infections, the primary germ cells are not infected, but some oocytes become infected; because only a proportion of the female gametes are infected the filial infection rate is <1. The ensuing reduction in prevalence rate can only be counteracted by amplification during the horizontal phase of transmission.

In this section the evidence for stable infections of arboviruses in mosquitoes is described and

contrasted with that for stable infections of sigma virus in *Drosophila*.

(i) *Maintenance of vertical-transmission rates*. There have been three reports of arboviruses being vertically transmitted through a number of generations of the mosquito host with little or no reduction in filial infection rate or vertical transmission rate.

(a) In experiments with La Crosse virus, the parental generation of Oc. *triseriatus* that was selected from a laboratory colony had a mean vertical transmission rate (71%) within the range measured in seven wild populations. Females of the parental generation were orally infected, and vertical transmission infection rates were determined in the F_1 to F_8 generation progeny. Over the eight generations, the vertical transmission rate among the female progeny varied between 51% and 88%, while that among the male progeny varied between 64% and 88%, showing no tendency to progressive increase or decrease (Miller *et al.*, 1977).

(b) Following intrathoracic inoculation of San Angelo virus into female *St. albopicta*, 46 vertically infected F_1 females were used to provide an F_2 generation, in which the filial infection rates ranged from 0 to 94.6% (fuller details in Section 44.6.3 above). Of the 46 F_1 mothers, five whose progeny showed filial infection rates of 83.3–94.6% were considered to have stable infections. A line was derived from a single F_1 female whose progeny had a filial infection rate of 88.7%. Filial infection rates among the progeny of F_3 and F_4 females were >93%, and among those of F_5 to F_8 females they were 100%. Eggs from a single female were used to start each of these generations, and in each case the parent female was from a family in which most or all of the members were infected (Tesh and Shroyer, 1980).

(c) Of six vertically infected adult female Oc. *dorsalis* reared from parents that had been intrathoracically inoculated with California encephalitis virus, only one F_1 female produced viable eggs, and viability was poor in the succeeding generations reared from that female. The filial infection rate for the F_2 and F_3 progeny (n = 38 and 8) was 100%, and for the F_4 progeny (n = 81) it was 91%. A single F_4 female produced F_5 progeny (n = 42), with a filial infection rate of 93% (Turell *et al.*, 1982d).

With regard to experiments of this sort, Fine (1975) pointed out that, where the experimental protocols involve selection of hereditarily infected hosts in each generation, any claims that high prevalence rates would prevail in natural populations are fallacious. This criticism is applicable to the three cases described above. It is possible that some of the females had stable infections, but it would be wrong to consider this a fact.

(ii) *Distribution in the body*. Typically, in orally infected females, virus appears first in midgut epithelial cells and disseminates later to other organs, always including the salivary glands. When observed, infection of the ovaries occurred later than in other organs, and most often within the ovariolar sheath and follicular epithelium, less commonly within oocytes (Section 44.8.1.f). The distribution of virus was examined in the vertically infected progeny of Oc. *triseriatus* parents that had been orally infected with LACV. In first- and second-instar larvae, viral antigen was detected in the fore-, mid- and hindgut and the Malpighian tubules. In third- and fourth-instar larvae it was present also in salivary glands and the CNS (central nervous system). The gonads were 'not available or distinguishable' until the late pupal stage, when antigen was detected in small amounts in the ovaries and testes. In adults, virus was present also in thoracic muscle, the accessory glands and gonoducts of males, and the oviducts of females. During metamorphosis, the larval salivary glands are histolysed and adult salivary glands develop from minute imaginal cells (Volume 1, Section 8.5). Fluorescent staining showed substantial viral antigen in the salivary glands of newly emerged adults, so vertically infected females should be able to transmit LACV during their first blood meal (Beaty and Thompson, 1976).

(iii) *Infection of ovarian tissues and cells*. A histological study was made of ovaries from *St. albopicta* females from a line that was 'chronically infected' with San Angelo virus; after 17

generations with vertical transmission some 75% of females were infected. In vertically infected females examined within 12 h of emergence, visualization by immunofluorescence showed virus to be present in most abdominal organs. In longitudinal sections, the ovaries were the brightest staining structures. At that stage of ovarian development (stage I), viral antigen was present in the ovarian and ovariolar sheaths, interstitial cells between the ovarioles and the oviduct. None was seen within the germaria, follicular epithelia, nurse cells or oocytes. In some follicles at stage II of ovarian development, viral antigen was seen only in the follicular epithelium; in others, it was visible in the follicular epithelium, nurse cells and oocytes. Following a blood meal and at stage III of ovarian development, when the oocytes were rapidly enlarging, the follicular epithelium stained intensely and there was a rapid accumulation of virus within the growing oocytes. In stage V a large amount of viral antigen was visible within the mature oocytes. It appeared that San Angelo virus entered developing follicles from the surrounding ovarian tissues. During a period of about 72 h, an impressive amount of viral antigen accumulated in the follicles, indicating virus replication. In some females, all mature oocytes within the ovaries were infected. In others, only 50–60% of follicles contained viral antigen, and the uninfected follicles were scattered randomly throughout the ovary, despite the presence of large amounts of viral antigen in adjacent follicles. Viral antigen was not seen within the germaria at any stage of ovarian development (Tesh and Cornet, 1981). This pattern of infection of ovarian tissues of *St. albopicta* with San Angelo virus contrasts with that in *Drosophila melanogaster* with stable infections of sigma virus: in all *Drosophila* embryos the primordial germ cells become infected, leading in the adults to infection of all female gametes and some male gametes.

(iv) *Hypersensitivity to carbon dioxide.* Adult *Drosophila melanogaster* that are infected with sigma virus fail to recover from brief exposure to CO_2 and die, whereas uninfected flies recover quickly from CO_2 anaesthesia (Section 41.3.2.a). Similar effects were seen in mosquitoes parenterally infected with California encephalitis virus. Within 3 days of inoculation with CEV, adult mosquitoes of ten species and four genera (*Anopheles*, *Culex*, *Culiseta*, *Ochlerotatus*) became hypersensitive to CO_2, failing to revive after 20 s exposure at 22°C. Uninfected mosquitoes quickly revived. Most or all infected individuals remained hypersensitive to CO_2 for up to 10 days after inoculation; the numbers that were hypersensitive then declined gradually to just a few by 30 days post-inoculation. In contrast, females of Oc. *dorsalis* vertically infected with CEV were not hypersensitive to CO_2, and neither were females inoculated with CEV. Inoculation of Cx. *tarsalis* with Main Drain or Turlock orthobunyaviruses had little or no effect on CO_2 sensitivity (Turell *et al.*, 1982b). Reports of induction of hypersensitivity to CO_2 by arboviruses other than species of *Orthobunyavirus* are summarized here by family.

Reoviridae. An orbivirus isolated from *Cx. quinquefasciatus*, and designated JKT-7400, induced hypersensitivity to CO_2 in *Cx. quinquefasciatus*, *St. albopicta* and *D. melanogaster* (Vazeille *et al.*, 1988).

Rhabdoviridae. Sigma virus induced CO_2 sensitivity in *Cx. quinquefasciatus* and *Tx. amboinensis*, but not in *St. albopicta*. Vesicular stomatitis New Jersey virus and three other species of *Vesiculovirus* induced hypersensitivity to CO_2 in *Cx. quinquefasciatus* and *Tx. amboinensis*, but vesicular stomatitis Indiana virus did not (Rosen, 1980). Mosquito species that, after inoculation with a virus, failed to recover after a brief exposure to CO_2 recovered quickly from anaesthesia with nitrogen or chloroform (Rosen, 1980; Vazeille *et al.*, 1988).

In conclusion, we note that infections of mosquitoes with some arboviruses show some similarities to infections of *D. melanogaster* with sigma virus, e.g. in the induced hypersensitivity to carbon dioxide. Three experimental results that purportedly showed stable infections were based, to a greater or lesser extent, on defective experimental design. Possibly some of the females had stable infections, but proof was not established. No evidence has been obtained of the infection of primordial germ cells in mosquitoes, a key aspect of stable infections of sigma virus in *Drosophila*. The

possibility of stable infections of arboviruses in mosquitoes deserves further investigation, and evidence of stable infections would have important implications for their epidemiology.

44.6.5 Falls in prevalence rate during phases of vertical transmission

For arboviruses that survive within vertically infected eggs of aedine mosquitoes, there are two possible types of infection. (i) Some or all infections are stable. The prevalence rate of infection remains much the same from one host generation to the next, and through any periods of adverse climatic condition. As noted above, only very limited laboratory evidence is consistent with the possibility that certain infections of arboviruses in mosquitoes are stable. (ii) All or most infections are non-stable. The prevalence rate of infection from vertical transmission declines from one generation to the next, and amplification during phases of horizontal transmission is needed to restore it.

With non-stable, mosquito-borne infections, there is always a fall in the prevalence rate of infection from that of the vertically infected, egg-laying females of one generation to that of the newly emerged adult females of the next because not all of the progeny of a female with infected germ cells become vertically infected. Consequently, the filial infection rate among the progeny of any infected female is <100%, and the vertical transmission rate among the progeny of the cohort of infected females is <100%. Laboratory experiments can show the extent of the fall. For example, when LACV was transmitted vertically through successive generations of Oc. *triseriatus*, a mean decline in prevalence of about 30% per generation was found (Miller *et al.*, 1977).

La Crosse virus, for which sciurid rodents are the amplifying hosts, and for which Oc. *triseriatus* is the main vector, is transmitted both vertically and horizontally. Its epizootiology has been investigated in some detail in the field, and estimates were available for the loss of prevalence during phases of vertical transmission. In a simulation model using quantitative data from field studies, the estimated extent of amplification was insufficient to compensate for loss of prevalence during vertical transmission, which might have been the result of a defect in the model or of incompleteness of data (Section 45.5.6.d).

44.7 SURVIVAL OF ARBOVIRUSES THROUGH CLIMATICALLY ADVERSE SEASONS

Most parts of the world experience a season of either prolonged low temperature or hot, dry weather during which arbovirus transmission ceases because the arthropod hosts have entered a state of quiescence or diapause and cannot serve as vectors. Culicids pass through adverse seasons as eggs, larvae or adults at various depths of dormancy. Where eggs or larvae are infected with an arbovirus, the infections have been acquired through vertical transmission. Where adult females are infected with an arbovirus, it will have been acquired either through vertical transmission or, in the case of females that have already taken a blood meal, through horizontal transmission. However, among the mosquito species in which conditions of day length and temperature can induce emerging adult females to enter a diapause state, most do not take blood meals either before or during this state.

44.7.1 Survival in or reintroduction by vertebrate hosts

Generally, once mammals or birds have become infected by an arbovirus, they remain immune to infection by that arbovirus for life, with the rare exception of host species in which a virus can persist in latent form for long periods within cells of the host, permitting eventual reactivation of the infection, or recrudescence. It is believed, therefore, that very few arbovirus species can survive in their mammalian or avian hosts through long, climatically adverse seasons. Further, new susceptible hosts are not born during adverse seasons. When the climate has become favourable again and the vector species reappear, the surviving warm-blooded hosts of almost all arboviruses are

uninfected or are immune and therefore not a source of virus.

(a) Survival in poikilothermic or hibernating vertebrates

Experimental evidence indicated that Western equine encephalitis virus might overwinter in poikilothermic animals. In initial experiments, adult garter snakes (*Thamnophis* spp.) were given intraperitoneal injections of WEEV and placed under natural winter conditions. Virus titres in the blood decreased progressively during the colder months, in most cases to apparent negativity, but they increased again to fairly high titres as temperatures rose in the spring (Gebhardt and Hill, 1960). Eighty-four snakes of three genera (*Thamnophis*, *Coluber* and *Pituophis*) which were captured between mid-May and mid-July 1963 in three swampy areas of Utah were lip tagged for identification and tested for WEEV; 37 of the snakes were naturally infected with the virus. When kept at room temperature through the summer (and tested several times) the infected snakes underwent periods of viraemia. Eight of the infected adult snakes were tested regularly until WEEV could not be detected, after which they were kept at 4–6°C for a month and then returned to room temperature. Twenty-one days later, three of the eight snakes were viraemic, with titres of 10^2 to 10^4 PFU ml^{-1}. During this study, vertical transmission of WEEV to a proportion of the young born to infected females occurred.

Females of *Cx. tarsalis* that fed on parenterally infected snakes acquired WEEV. Females that became infected with WEEV through feeding on infected chicks transmitted the virus to seven of 12 snakes offered as blood sources, and the infected snakes developed viraemias of up to 2.9×10^7 PFU (Gebhardt *et al.*, 1964, 1966). Reviewing these findings, Gebhardt and Stanton (1967) concluded that WEEV can overwinter in snakes, that infected snakes can become viraemic during the spring, and that mosquitoes might then transmit the virus from snakes to birds. This remains to be confirmed in the field. *Culex tarsalis*, the main vector of WEEV, is an opportunistic feeder, attacking birds, mammals and reptiles (Volume 2, Section 39.3.1.d).

It has been postulated that bats can become chronically infected with Japanese encephalitis virus. Two strands of evidence from wild populations in Japan may support this view: (i) the prevalence rate of JEV in insectivorous bats remained about the same during all seasons of the year; and (ii) JEV was isolated from a bat that was seropositive for anti-JEV neutralizing antibody. Experimental evidence from the North American bat *Pipistrellus subflavus* was consistent with that view. JEV was not detectable in blood samples from bats kept for up to 107 days at 10°C, but 3 days after return to room temperature the bats became viraemic (Section 45.4.4.c).

(b) Recrudescence in birds

A number of viruses, notably HIV and bluetongue virus, persist in latent form for long periods within cells of their vertebrate hosts, permitting the infection eventually to break out again. Investigations have been undertaken, mostly in California, to find whether certain arboviruses can survive through the winter in the tissues of infected birds, and whether latent infections might recrudesce in the spring, causing the birds to become viraemic. Positivity in RT-PCR tests revealed the presence of viral RNA but not of infectious virus.

Over a number of years, the possibility was examined that WEEV or SLEV might persist in house finches (*Carpodacus mexicanus*) through the Californian winter and recrudesce in the spring. House finches are year-round residents and hosts of those arboviruses. Neither virological tests nor xenodiagnostic assays on wild-caught birds provided evidence of natural relapse with WEEV or SLEV. House finches caught at Bakersfield were kept in an outdoor aviary, inoculated with WEEV or SLEV, and the titres and durations of viraemia recorded. Assay by PRNT showed the WEEV-specific antibodies persisting well, blocking viraemia in birds reinoculated after 6 months, and remaining at high titre after 10 months. In contrast, within 6 months, SLEV-specific antibodies had declined in half of the

test birds (Reisen *et al.*, 2001). When a year after infection house finches were challenged with the same strain of SLEV, there was a rapid and marked rise in titres of SLEV-specific antibodies, and the virus was not detectable in blood samples taken on days 1–4 post-challenge or by xenodiagnosis. Reisen *et al.* (2003, 2004) concluded that the protective antibodies that are induced in house finches by infection with WEEV or SLEV during one season persist, preventing reinfection with homologous virus during the following season.

Hatching-year grey catbirds (*Dumatella carolinensis*) captured in Ohio during the summer 2006 were inoculated with WNV during September. Upon necropsy at 24 weeks post-infection, no viral RNA was detected in the kidney, spleen, brain or liver (Owen *et al.*, 2010).

When 82 birds of eight species were inoculated with WNV and necropsied >6 weeks later, one or more organs in 28 of the birds were positive for WNV RNA. Among 23 house finches, 12 were positive in RT-PCR tests on spleen, lung or kidney, while four of six positives were shown to contain infectious or potentially infectious WNV. WNV RNA was detected in serum from a single house sparrow. Reisen *et al.* (2006b) concluded that WNV RNA persists within the organs of several species of birds but does not circulate frequently within the bloodstream. On a longer time scale, WNV-inoculated house sparrows were monitored for persistent infection for up to 2 years. Infectious virus persisted in tissues through 43 days, but not in sera beyond 6 days. Viral RNA persisted in tissues through 65 days. Nemeth *et al.* (2009b) concluded that the chronicity of WNV infection in some tissues, but not blood, was consistent with infection by predation during overwintering, but not with recrudescence.

(c) Annual reintroduction by migratory birds

It has been suggested that, after winter has ended, arboviruses might be reintroduced into their areas of summer endemicity by newly arrived, infective migrating birds. By this means, local vectors could become infected, and in turn transmit the virus to local avian hosts. The only well-supported example of reintroduction is that of **West Nile virus** transported by white storks (*Ciconia ciconia*) during their autumn migration from central Europe to Africa. Infectious WNV was isolated from injured birds in a flock of white storks forced by the weather conditions to land in Israel during their migratory flight. Migrating juveniles could have become infected while resting, en route, in an area of WNV endemicity such as the Danube delta (Section 45.7.8.b). This is the most persuasive evidence available of the transport of an arbovirus by migrating birds.

The carriage of **Japanese encephalitis virus** by infected, migratory ardeid birds flying from areas in the southern hemisphere to Japan has been suggested as an explanation for the sudden infection with JEV of farmed swine or populations of *Culex tritaeniorhynchus* in cool-temperate regions of Japan during the spring or early summer (Ogata *et al.*, 1970; Endy and Nisalak, 2002). However, the annual timings of first reappearance of JEV in Japan after the winter are inconsistent with its introduction by returning ardeids (Section 45.4.7.e).

During the summer and early autumn of 1965, both **Eastern** and **Western equine encephalitis virus** were active on the Atlantic seaboard of the USA, infecting many birds. From August to October, 1506 birds trapped at locations from the eastern shore of Maryland to southern Florida were screened for arboviruses. They were classed as either resident species or migrant species, but some of the latter were not necessarily migrating at that time. EEEV was isolated from 16 of the 1506 birds screened, and WEEV from two, the hosts being 14 species of birds caught while migrating southwards along the Atlantic Flyway (Lord and Calisher, 1970). The authors commented that the postulated northward and southward transport of EEEV by birds was inconsistent with the antigenic differences between the North American and South American strains of that virus.

In a later investigation of the northwards migration of birds over the eastern seaboard of the USA each spring, Calisher *et al.* (1971) tested the postulate that some of the migrants might become

infected with EEEV while en route, possibly in Florida or the Gulf Coast. A South American serotype of EEEV was detected in two birds captured in the Mississippi delta (Louisiana), which were thought to be migrating northwards – one a blackpoll warbler (*Dendroica striata*) and the other a wood thrush (*Hylocichla mustelina*). This was considered evidence of the transport of EEEV northwards across the Gulf of Mexico (Calisher *et al.*, 1971), but later genetic findings were inconsistent with that conclusion. Nucleotide sequencing and phylogenetic analyses showed that North American isolates of EEEV constitute a single, highly conserved lineage (lineage I), whereas South American isolates group into three distinct lineages (II–IV). It is possible that lineage I strains of EEEV recovered in Mexico and Caribbean islands were introduced by birds (Section 45.1.1).

44.7.2 Reintroduction by mosquitoes carried on wind

It has been well established that mosquitoes are carried by winds at heights above the boundary layer (Volume 2, Section 33.9.3). The carriage of *Culex annulirostris* in wind over New South Wales, and of *Cx. tritaeniorhynchus* in wind over the East China Sea are mentioned briefly in Sections 45.4.2.c and 45.4.7.b, respectively. The possibility that *Culiseta melanura*, a main vector of EEEV, might be carried by winds from the southern USA northwards was investigated by using past meteorological maps to analyse earlier wind flows over the Eastern Seaboard. In five southern states, *Culiseta melanura*, a main vector, is active all through the year, and in Florida EEEV has been isolated from and the disease seen in horses in each month of the year.

The atmospheric pressure at mean sea level is approximately 1000 millibars (mb), and it decreases logarithmically with height. Six-hourly surface-level maps and 12-hourly 850 mb maps were scrutinized for atmospheric pressure, wind direction and speed, temperature, precipitation and weather fronts. The backward trajectories of the winds were computed every 6 h for 5 days, starting at three levels: 1000, 900 and 850 mb at approximate heights of 0.1, 1.0 and 1.5 km above sea level, respectively. Possible sources of airborne mosquitoes were determined from the backward trajectories every 6 h up to 30 h to correspond with possible mosquito flight times. Convergence (of air streams) and rain are associated with the passage of cold fronts, and convergence is known to lead to the concentration of airborne insects. The trajectory analyses suggested that mosquitoes could have been carried on surface winds at temperatures of 13°C or higher from North Carolina north-eastwards along the Atlantic Coast to Maryland and New Jersey, and thence to upstate New York, and from western Kentucky to Michigan (Sellers and Maarouf, 1990).

The landing of insects carried in an air stream is associated with convergence of the air stream with a cold front and rain. The irregularity of such events results in variations, from year to year, in the location and timing of insect landings and of any subsequent outbreaks of disease. Unfortunately, there were no past records of the dates of first appearance in spring of adult *Cs. melanura* that could not be explained by their development from overwintering larvae.

An examination of surface weather maps from 1982 to 1985 showed that the earliest days on which Eastern equine encephalitis was observed in horses, the virus isolated, or antibody detected were preceded by one or more days on which S to SW winds met cold fronts approaching from the north-west over New Jersey and the adjacent Delmarva Peninsula. The 1000 mb trajectories indicated that North Carolina could have been the source for the Delmarva Peninsula within 8–18 h, and that Delaware, Maryland, Virginia and North Carolina the sources for New Jersey within 4–24 h. The intervals between the possible arrival of EEEV and disease in horses were 5–11 days, and between the possible arrival of EEEV and detection of virus or antibody 1–8 days. The air temperatures were 13°C or higher, and in ten out of 11 instances of possible arrival coincided with the passage of a cold front and rain; in the remaining instance arrival coincided with rain. Sellers and Maarouf (1990) came to the preliminary conclusion that

EEEV could have been carried on surface winds by infective mosquitoes, which on arrival fed on birds. They commented that such introductions of EEEV would not lead to infection in horses if bridge vectors and their hosts were not present early in the year.

44.7.3 Survival in vertically infected eggs or larvae

With aedine mosquitoes, it appears to be in the egg stage that populations may be exposed to long periods of adverse climatic conditions, whether of heat or cold. In fact, embryonic development starts immediately after oviposition and continues to completion, and what appear to be dormant eggs are eggshells occupied by pharate first-instar larvae. Almost all aedine species lay desiccation-resistant eggs that can persist and survive for long periods away from free water – those of some species for months, of other species for over a year. This enables them to hibernate or aestivate.

Only a very small number of non-aedine mosquito species are known to be able to maintain an arbovirus infection while surviving winters in the larval stage. One is *Cx. erythrothorax* infected with WNV (Section 44.6.2.b); however, the evidence presented is insufficient to demonstrate a contribution of vertical infection to perpetuation of the virus in this host. (See also Subsection 44.7.3.d below.)

(a) La Crosse virus

A well-documented case of survival of an arbovirus within vertically infected aedine eggs is that of La Crosse virus, a strain of *California encephalitis virus* (family *Bunyaviridae*) in the eggs of *Ochlerotatus triseriatus* (Section 45.5.5). This is a tree-hole mosquito which at higher latitudes overwinters only in the egg stage. In Wisconsin, where winters are hard, vertically infected eggs laid during the autumn of one year hatch during the following spring, giving rise to infected adults. That LACV overwinters in diapausing eggs of *Oc. triseriatus* is known from its presence in larvae that hatch from post-diapause eggs.

A study of LACV infections in the post-diapause larvae and adults of *Oc. triseriatus* was undertaken in upland, oak/hickory forests near La Crosse (Wisconsin; c. 43° 83' N), an area where LACV is endemic, infecting squirrels and chipmunks. During the early spring of 1974, 103 water-filled tree holes likely to be oviposition sites were marked and characterized. The tree holes were sampled weekly from 30 April to 17 June, and then biweekly from 24 June to 24 September. They were sealed with aluminium mesh on 14 June, prior to adult emergence. Pupae were first detected in the tree holes on 16 June, and the first adults were found on 25 June.

Of the 103 marked tree holes sampled before adult emergence, 64 contained larvae (n = 1698). Of those 64 tree holes, 55 yielded only uninfected larvae (n = 1188), while nine (n = 510) yielded ten larvae positive for LACV (7♂♂ and 3♀♀). The MIR for larvae from the infected tree holes was 19.6/1000, while that for the whole sampled larval population was 5.9/1000. A more equal sex ratio of infection was obtained when all individuals from 12 tree holes in a particular area (GW) and collected between 30 April and 31 August 31 were tested. Seven tree holes (n = 299) provided no infected individuals, whereas five tree holes (n = 981) provided 16 infected individuals. Of those 16 individuals positive for LACV, nine were male and seven were female – 56.2% and 43.8%, respectively. The MIR for all 12 tree holes was 12.5. Beaty and Thompson (1975) considered that the 16 infected mosquitoes would have been 'adequate to reinitiate the seasonal arthropod-mammal cycle in the area'. LACV was isolated from some *Oc. triseriatus* emerging in enclosed tree holes throughout the summer months, demonstrating that these sites allow not only the overwintering of LACV but also its continuing release through the summer season in this forested area.

Apparent evidence of amplification of LACV was obtained when higher MIRs were measured in wild-caught adult *Oc. triseriatus* than in larvae and pupae collected from tree holes. However, mathematical

analysis of theoretical host and vector populations that were given the characteristics of natural populations in an area endemic for LACV produced an estimate of the extent of amplification during phases of horizontal transmission that was insufficient to compensate for the fall in prevalence rate that occurs during all phases of vertical transmission (Section 45.5.6).

(b) Keystone virus

Ochlerotatus atlanticus, the vector of Keystone virus (KEYV), has just one generation per year and overwinters in the egg stage. The isolation of KEYV from wild-caught larvae of *Oc. atlanticus*, and from males and females reared from wild-caught larvae, confirmed that vertical transmission occurs in nature. The first isolation of KEYV from wild adults occurred on virtually their first appearance in spring, when the females were unlikely to have taken a blood meal; therefore, the adults of the vector population must have been vertically infected at the time of emergence (Section 41.4.2.a).

(c) Rift Valley fever virus

In eastern and southern Africa, Rift Valley fever virus (*Phlebovirus*, family *Bunyaviridae*) can survive through long dry periods within vertically infected *Neomelaniconion mcintoshi* eggs laid at the edge of large to huge ground-surface pools, which will become flooded when prolonged heavy rainfall raises the level of the water table. It appears feasible that, after the flooding of the pools and the later emergence of adults in massive numbers, epidemics of Rift Valley fever among young farmed ruminants can be initiated by the bites of vertically infected *Ne. mcintoshi* (Section 45.6.4.c). However, this remains to be demonstrated.

(d) West Nile virus

In certain investigations into whether or not WNV might survive through the winter in diapausing mosquitoes, only the standard RT-PCR or TaqMan RT-PCR tests, which detect viral RNA, were used (Phillips and Christensen, 2006 – in larvae; Cupp *et al.*, 2007 – in overwintering adults). Those tests were not supplemented by PRNT tests (Section 44.3.4.g), which would have indicated the presence or absence of infectious viral particles, so allowing the drawing of possibly erroneous conclusions Other investigators who had identified WNV-RNA positive pools by RT-PCR tests continued by testing the positive pools for infectious virus. Of 91 pools produced from overwintering female mosquitoes collected in New York City, three pools, all containing extracts of *Culex* spp., tested positive for WNV RNA by TaqMan RT-PCR, but only one proved positive for infectious WNV virus particles when inoculated into Vero cell culture (Nasci *et al.*, 2001). One of 75 pools formed from *Cx. pipiens* collected while overwintering in Pennsylvania was positive for WNV by RT-PCR, but in Vero cell culture it was negative (Bugbee and Forte, 2004). Adult female *Cx. p. pipiens* collected while overwintering in New Jersey were kept for 6–21 days under diapause-breaking conditions. One pool out of 34 proved positive for WNV RNA when tested by TaqMan RT-PCR, but infectious virus particles could not be detected by Vero cell plaque assay or by inoculation into a culture of C6/36 mosquito cells (Farajollahi *et al.*, 2005).

44.7.4 Survival in vertically infected adults

(a) Introduction

Once an adult mosquito has become infected with an arbovirus, the infection persists throughout its remaining lifespan (Section 44.8.1.g). But is that the case with females physiologically induced to enter diapause which were infected through vertical transmission or by feeding on infected hosts before entering or when in hibernacula?

The survival of adult mosquitoes through a long period of diapause requires the provision of reserves, obtained by feeding before entering hibernacula, or while within hibernacula. It also requires an appropriately programmed reproductive

physiology. Females of *Culex pipiens pipiens*, *Cx. tarsalis*, *Culiseta inornata* and *Anopheles messeae* feed on plant juices to build up their lipid reserves before entering hibernacula, and do not blood feed until after completing diapause (Hudson, 1979; Mitchell, 1981; Jaenson, 1987). Females of a few species take blood meals during the winter. Females of *Anopheles atroparvus* that are in the proximity of farmed cattle blood feed before entering cattle sheds as hibernacula, and feed periodically through the winter. Females of *Anopheles sacharovi* leave their hibernacula to blood feed. These females are physiologically programmed to dissociate ovarian development from blood feeding while in diapause, a condition termed gonotrophic dissociation, and they remain gonoinactive until shortly before the diapause state ends. Species that employ this strategy could acquire arbovirus during one season and transmit it during the next (Swellengrebel, 1929; Mer, 1931; de Buck *et al.*, 1933).

Turell (1988) postulated that, in cool-temperate regions, overwintering females of *Culex pipiens*, *Cx. tarsalis* and *Cx. tritaeniorhynchus* serve as amplifying hosts of arboviruses, which they can transmit after emerging from diapause in the spring. Each of those three *Culex* species occurs over a substantial geographical range, and their physiology and behaviour differ at different latitudes. For that reason it is necessary to consider separately the possible overwintering of viruses at latitudes where winters are severe and where they are mild.

(b) Survival through severe winters

Females of the spring or early-summer generations of all or most species of *Culex* do not survive beyond the autumn. The generation that yields adults in late summer or autumn passes through its aquatic stages at a time of shortening day length and cool or moderate temperature, and the adult females that emerge are programmed for diapause. Characteristically they are non-responsive to host cues, are gonoinactive, and enter hibernacula after building up reserves from sugar feeding (Mitchell, 1981, 1983). Populations of all three of the *Culex* species named above have been sampled during the winter months in searches for infected females. At higher (northern) latitudes, most investigations produced negative results, e.g. the absence of Japanese encephalitis virus from some 99,000 overwintered *Cx. tritaeniorhynchus* captured in the Nagasaki area between March and late May (Table 45.17). Positive results were obtained from two early investigations. (i) In Colorado, at *c.* 40° N, a single isolate of WEEV was obtained from hibernating *Cx. tarsalis* (n = 1361) (Blackmore and Winn, 1956); and (ii) two isolates of SLEV were obtained from females of *Cx. pipiens* caught in Maryland during January and in Pennsylvania during February, both months of subzero mean minimum temperature. Both of these females had been kept at laboratory temperature for 2–3 weeks before examination (Bailey *et al.*, 1978).

The virological assays used by some early investigators might have been insufficiently sensitive to detect viruses that were present. Infections with WEEV in diapausing *Cx. tarsalis* were maintained at levels undetectable by plaque assay or Vero cell culture, but were detected by RT-PCR (Reisen *et al.*, 2002). West Nile virus was not detected in pools of wild-caught *Culex* spp. when tested by traditional RT-PCR, but positive results were obtained with TaqMan RT-PCR assays (Nasci *et al.*, 2001).

Following an outbreak of West Nile fever in New York in 1999, some 2383 mosquitoes, almost entirely *Culex pipiens*, were collected from hibernacula (located at *c.* 40° 44' N and 40° 50' N) during January and February 2000 and grouped into 91 pools. Three pools were positive when tested by TaqMan RT-PCR, showing the presence of WNV RNA (MIR = 1.3), and live virus was isolated from one of the three pools (Nasci *et al.*, 2001). At a location in Pennsylvania, a variety of possible hibernacula were searched for overwintering mosquitoes from December to April in two winters: 2001–02 when 724 *Cx. pipiens* were collected; and 2002–03 when 501 *Cx. pipiens* were collected. At the times of collection, the average temperature within the hibernacula was 1.7°C. Assay by TaqMan RT–PCR found no WNV in the first collection, but it was present in one of 27

pools (MIR = 2.0) from the second (Bugbee and Forte, 2004).

In Stratford, Connecticut (41° 10' N), during September 2005, the weekly MIRs for WNV in adult female *Cx. pipiens* were 13–43. Females were collected, blood fed and allowed to lay, and each female that oviposited was tested for WNV. The egg rafts from ten infected females were allowed to develop, and the F_1 larvae from those egg rafts were reared under conditions of day length and temperature that would later induce a diapausing state in the emerging adult females. Shortly after emergence of the F_1 adults, all the males and five females were tested for WNV; the remaining females were kept for ~140 days under conditions that maintained diapause. After that period they were transferred to non-diapause conditions and allowed to feed on a hamster. None of the F_1 adult male progeny (n = 425) from naturally infected parental-generation females were infected with WNV. Of the F_1 adult female progeny that were tested (n = 288), two were infected – offspring of the same female. The filial infection rate among the 30 F_1 progeny from the female parent numbered T676 was 21.3/1000, but if calculated for the F_1 offspring from all ten infected parents it would have been 2.8/1000. One of the two F_1 females infected with WNV was fed on an uninfected hamster 167 days after emergence. The hamster died 8 days after being bitten, when WNV was detected in its tissues (Anderson and Main, 2006).

(c) Survival through mild winters

Descriptions have been published of the persistence of Japanese encephalitis virus and of Western equine encephalitis virus through mild winters. The varying fate of JEV during milder and less mild winters in islands of the Ryukyu archipelago, where *Cx. tritaeniorhynchus* is the main vector and domesticated pigs are the amplifying host, is described in Section 45.4.7.b.

In North America, birds are the amplifying hosts of WEEV, and *Cx. tarsalis* is the main vector. The duration of winter diapause in *Cx. tarsalis* shortens from 6 months in Canada to 2–3 months in the southern part of Central Valley, California, and to 1 month in the Coachella Valley of southern California (Anderson and Harwood, 1966; Nelson, 1971; Reisen *et al.*, 1995). In the Lower Rio Grande Valley of Texas (c. 26° N), *Cx. tarsalis* does not have a winter diapause (Eads, 1965).

In Kern County, California (c. 30° 20' N), WEEV could be isolated from *Culex tarsalis* at all times of year except for the 2-month period from mid-November to mid-January. The periods when WEEV could and could not be isolated from *Cx. tarsalis* correlated with periods of blood feeding and diapause, respectively (Reeves *et al.*, 1958; Bellamy and Reeves, 1963). Experiments showed that WEEV could be maintained through the winter in female *Cx. tarsalis* if they fed on a viraemic host in the autumn, survived to the end of diapause, and fed on a susceptible host in late winter or spring. However, field studies undertaken in Kern County over a number of years indicated that this must be a rare event under natural conditions. It was more likely that post-diapause vectors found after mid-January to be infected had recently fed on a viraemic host (Reeves, 1971). Analyses by RT-PCR detected no infections with WEEV in female *Cx. tarsalis* (n = 917) collected in Kern County during the winter following a summer with enzootic WEEV activity (Reisen *et al.*, 2002).

(d) Survival through hot, dry seasons

In different parts of West Africa, YFV appears to persist throughout long dry seasons, when the larval habitats have dried out and adult mosquitoes are not found. On the strength of a few reports of vertical transmission in wild mosquito populations of YFV (Section 45.3.5.b) or DENV (Section 45.2.7), authors have proposed that vertical transmission in dormant aedine eggs provides the means of survival and perpetuation of the viruses in those locations. None have attempted to substantiate the hypothesis by developing a simulation model from their field data, possibly

because so much supplementary information would be needed.

Circumstantial evidence suggests that yellow fever virus may survive through hot, dry seasons within adult female hosts. Three cases are of interest. First, in the Rivas region of Nicaragua, during April and May 1953, towards the end of the dry season, there was an almost total absence of mosquitoes, including *Haemagogus* spp., the known vectors. Yet, in spite of the apparent absence of mosquitoes during 5 or 6 months of intense drought, as soon as the rains started monkeys began to die of yellow fever at the exact point reached by the epizootic wave when the dry season started (Boshell, 1955) (Section 45.3.6.f).

Second, in the forested great plains of eastern Colombia, a severe dry season occurs during January and February; exceptionally, it may last for 4 months. At La Cuchilla, during the dry season of 1941, when *Haemagogus janthinomys* was rarely observed at ground level it could be captured in forest at 7–15 m elevation. This mosquito was caught in substantial numbers during January, in moderate numbers during February and March, and in substantial numbers again in April. Yellow fever virus was isolated from females captured in the canopy in mid- and late January. A single apparent case of transmission was reported. In early February 1941, an unvaccinated man felled a tree not more than 20 m from a site where, on 31 January, three infected females had been caught. A week later he became sick, and died of yellow fever. The original source of the virus must have been the animal population – mostly marsupials and a very few monkeys. On this evidence, Bugher *et al.* (1944) concluded that *Hg. janthinomys* can survive through the dry season in the forest canopy, and postulated that YFV can survive from one rainy season to the next in such mosquitoes (Section 45.3.6.g).

Third, in a forested region of Panama near Pacora, where yellow fever was endemic, adults of *Sabethes chloropterus* (another vector of YFV) were present all year round. Their eggs do not withstand desiccation (unlike those of *Haemagogus*). The numbers of adult *Sa. chloropterus* reached a peak during the rainy season in July to September, decreased during October and November, and fell sharply with the onset of the dry season in January (a time when no adults of *Haemagogus* could be found). Galindo *et al.* (1951, 1955) surmised that the small population of adult *Sa. chloropterus* present during the dry season might have come from surviving larvae. The larvae occupy tree holes of a characteristic form: a small opening gives entrance to a relatively large, flask-shaped rot hole, which holds water throughout the dry season.

44.8 INFECTION, REPLICATION AND DISSEMINATION IN MOSQUITOES

During a transmission cycle, arboviruses must be able to replicate alternately in the different internal environments of their vertebrate and arthropod hosts, and within each host type the infection must develop in such a way that virions can be transmitted to the other through the bite of a vector.

Some mosquito species, or strains, have heritable characteristics that make the females susceptible to infection and effective vehicles for transmission of an arbovirus from one vertebrate host to another. These characteristics have the nature of permissiveness to 'actions' of the arbovirus, i.e. permissiveness to (i) invasion of cells of the midgut epithelium and replication within them; (ii) dissemination into the haemocoel, permitting carriage in the haemolymph; or (iii) invasion of cells of the salivary glands, replication within them, and entry into the salivary ducts. The extent of the permissiveness of a mosquito species or strain to a particular arbovirus can be quantified in the laboratory as 'vector capacity', i.e. the proportion of a batch of mosquitoes that, having taken an infectious blood meal, subsequently can transmit the arbovirus to a susceptible vertebrate host. Working in the laboratory with *Cx. pipiens* that had imbibed RVFV or WNV from a viraemic host, Turell *et al.* (1984a, 2006) distinguished females in which the virus disseminated from those in which it did not by screening the legs and body separately. If virus was

recovered from the body but not the legs, the mosquito was considered to have had a non-disseminated infection limited to its midgut.

Many mosquito species that show high vector capacity for a particular arbovirus in the laboratory may never transmit it in nature, or may not have the characteristics necessary to be a 'subsidiary' or a 'main' vector. In the field, factors such as likelihood of contact with vertebrate hosts and host preference determine which sympatric, vectorially competent species serve as vectors (Section 41.1.3). These factors govern the 'vector potential' of vectorially competent species.

Species of *Ochlerotatus*, *Stegomyia* and *Culex* predominate among the known natural vectors of mosquito-borne viruses. No anopheline has been fully established as an arbovirus vector, but a few are putative vectors. *Anopheles funestus* is the putative main vector of O'nyong-nyong virus (*Togaviridae*), and *Anopheles gambiae s.l.* is a putative subsidiary vector, but it has been suggested that the virus may be isolated from it only during epidemics (Corbet *et al.*, 1961; Williams *et al.*, 1965; Lutwama *et al.*, 1999). Orungo virus and Banna virus (*Reoviridae*) have been isolated from species of *Anopheles* (Section 44.1.3). In areas of uncertainty such as this, only evidence from wild populations, or from the very earliest colonized generations, is relevant.

All studies of infected mosquitoes described in this section were undertaken on orally infected, not parenterally infected, females. In most instances, the mosquito species were natural hosts of the virus used. In most, possibly all, instances, the mosquitoes were not from the wild, or an early filial generation, but from long-established colonies.

44.8.1 Characteristics of infection, dissemination and persistence

Arboviruses of the families *Togaviridae*, *Flaviviridae* and *Bunyaviridae* that are discussed in this section all replicate in host cell cytoplasm. The midgut is the site of infection by arboviruses and of their initial replication. The salivary glands are a site of further replication and, through their ducts, of transmission to vertebrates.

(a) Virus intake

The epithelia of the foregut and hindgut are lined with cuticle, that of the midgut is not. Apart from the short bulbous cardia at its anterior end, the midgut consists of a narrow tube-like anterior region through which blood passes, and a flask-shaped posterior region which retains the blood meal and which in culicines is capable of much distension. Within a few minutes of feeding, the erythrocytes are clumped into a tight mass, or bolus, as a result of the extraction of water from the blood meal through diuresis. The bolus is surrounded by a narrow layer of cell-free serum (Volume 1, Sections 13.2, 14.3.4).

Little is known of the distribution and immediate fate of arbovirus particles within the bolus. At 1, 4 and 8 h after *Cx. pipiens* fed on mice viraemic with SLEV, virus particles were seen by TEM (transmission electron microscopy) in very small numbers within the blood bolus. They were dispersed among the erythrocytes, and only rarely were any seen near the apical margin of the epithelial cells. In one instance, at 8 h after feeding, virus particles were seen within vacuoles in single midgut epithelial cells. After 8 h, virus particles could no longer be seen within the gut contents. A peritrophic matrix was not present during the first 12 h after feeding (Whitfield *et al.*, 1973). Virus particles near the outside of the blood bolus or between the bolus and the midgut epithelium would risk attack by digestive enzymes. In *St. aegypti*, early trypsin peaks at about 2.5 h after feeding (Noriega and Wells, 1999).

When *Culex taeniopus* fed on chicks viraemic with Western equine encephalitis virus, imbibing amounts that would infect all females ($10^{5.0–5.4}$ PFU), small clusters of virions were seen only infrequently within the blood bolus at 0–4 h post-blood meal, and were never seen in contact with the apical surface of the midgut epithelium (Weaver, 1986).

The amount of virus that is imbibed when susceptible females feed on a viraemic host affects the likelihood of infection. Thus, among batches of *Oc. triseriatus* that fed on hamsters viraemic with La Crosse virus, at titres that increased in five steps from $10^{2.0}$ to $10^{4.6}$ $TCID_{50}$ (50% tissue culture infective dose) ml^{-1}, the percentages that became infected and later transmitted the virus to suckling mice increased progressively from 36% to 100% (Watts *et al.*, 1972). A blood meal of 5 mg containing LACV at the lowest titre of the range cited above would contain only 0.5 $TCID_{50}$, so LACV is very efficient at infecting its natural vector.

(b) Leakage

Each columnar cell of the midgut epithelium is strongly connected to its neighbours by regions of attachment of different types – continuous junctions, gap junctions, septate junctions or spot desmosomes. These provide the mechanical strength needed to withstand the substantial distension of the midgut that occurs in engorged females (Volume 1, Section 13.2.1). Ultrastructural examination of thin sections from midgut cells of *Cx. tarsalis* that had taken a blood meal showed the presence between columnar cells, and on the basal lamina, of blood components – possibly haemoglobin. Houk and Hardy (1979) described this as 'leakage', and suggested that leakage accounted for reports of rapid invasion of arbovirus into the haemocoel.

In one such case, after females of *St. aegypti* had fed through a membrane on blood containing Uganda S virus (*Flavivirus*, family *Flaviviridae*), haemolymph was withdrawn through a fine glass capillary inserted into the heart. Of 14 haemolymph samples taken after intervals of 5 min to 7 h after feeding, three contained virus. They had been withdrawn at 10 min, 30 min and 2 h after feeding, and contained virus at titres of 0.01, 0.01 and 0.13%, respectively, of the total body load (Boorman, 1960). A similar experiment was undertaken after imbibition of Whataroa virus (*Alphavirus*, family *Togaviridae*) by females of *Halaedes australis*, not a natural host. In three of 27 females sampled between 30 min and 4 h after feeding there were 'very substantial amounts of virus in the haemolymph' (Miles *et al.*, 1973).

(c) Invasion of alimentary canal cells

The presence of many particles of WEEV in the basal region of midgut epithelial cells of *Cx. taeniopus* 3–4 h after engorgement suggested that entry and replication had occurred by that time, when also many virions were visible on both sides of and within the basal lamina (Weaver, 1986).

With some mosquito/virus combinations, intake of infected blood results in invasion of only very few midgut epithelial cells. In *Cx. pipiens* infected by SLEV, even after 32 days no more than one in five cells had been invaded (Whitfield *et al.*, 1973). The sites of infection with O'nyong-nyong virus (ONNV) in *An. gambiae* were identified by use of a recombinant ONNV genome in which a GFP (green fluorescent protein) gene had been inserted. Females were fed on suspensions of ONNV in blood at a lower dosage of 5–6 $\log_{10}$ or a higher dosage of 6–7 $\log_{10}$ PFU ml^{-1}. On days 4 and 7 post-infection, a typical infection pattern with the lower dosage was of one to three infection foci in the posterior midgut epithelium, mean 1.5 foci, combined with infection in the anterior midgut. Not until days 10–14 did a more dispersed infection pattern in the midgut appear in a few females. Females fed the higher dosage showed the same pattern of infection, but with an average of 2.8 foci/midgut (Brault *et al.*, 2004).

Binding to a specific receptor is the first step in the penetration of cells by virus. A binding assay for ^{3}H-labelled DENV-2 with Scatchard plot analysis was used to examine the kinetics of early interactions of DENV-2 with cells from two mammalian cell lines (LLC-MK_2 and Vero) and two mosquito cell lines (C6/36 and AP61). In all cases, DENV-2 binding to cell surfaces was specific and saturable, indicating a finite number of receptors. The number of binding sites per cell ranged from 1.4 to 3.5×10^4 on the mammalian cells and from 4.3 to 6.6×10^3 on the insect cells.

The cell-surface receptors had a high affinity for DENV-2, with dissociation constants (K_d) in the range 142–171 pM for the mammalian cells and 98–125 pM for the insect cells. The pairs of mammalian and mosquito cell lines were shown to be distinct: treatment with neuraminidase significantly reduced binding to the mammalian cells but not to the mosquito cells, while heparin competed with DENV-2 for binding to the mammalian cells but not to the mosquito cells. This suggested that the mammalian cell-surface receptor contains sialic acid and heparin sulphate, whereas the mosquito receptor does not. Thaisomboonsuk *et al.* (2005) surmised that the DENV envelope protein may have two receptor binding domains, one that recognizes heparin sulphate/sialic acid moieties on mammalian cells and another that recognizes an unidentified moiety on mosquito cells. The binding of LACV to receptors is described in Section 44.8.2.b.

Replication of SLEV was first seen in midgut epithelial cells of *Cx. pipiens* at 6 days after feeding. Between days 6 and 8 post-feeding the virion density within those cells increased, and between days 9 and 12 the number of infected cells increased. Mature enveloped virus particles occurred primarily within the cisternae of the endoplasmic reticulum. Extracellular virions could be seen in narrow spaces between adjoining epithelial cells and, increasingly, between the basal cell membrane and the surrounding basal lamina. Some were located between layers of the basal lamina (Whitfield *et al.*, 1973).

(d) Dissemination

In most studies of dissemination, the distribution of viral antigen was discerned by direct immunostaining of whole organs or sections. Electron microscopy provided further information. The speed of dissemination varied in different mosquito/virus combinations.

In *Cx. pipiens* infected with RVFV and kept at 26°C, virus was found beyond the midgut in 6% of females at 12 h, in 9% at 24 h and in 22% at 48 h post-blood meal (n = 100, at each time). In a second study, 12% of females had virus beyond the midgut at 4 h post-blood meal (n = 50) (Faran *et al.*, 1988). When females of *Cs. melanura* kept at 25–30°C were fed on chicks viraemic with EEEV, the whole-body virus load increased from day 1 to day 7, and then gradually decreased until day 26. Virological assays indicated that the midguts were infected after 1 day, and the hindguts after 2 days when also most females contained detectable virus in their haemolymph. The apical surface of the hindgut epithelium is permanently protected by a layer of cuticle, so the presence of virus particles within those cells suggested invasion from the haemocoel and through the basal cell membranes. Figure 44.6 shows virus particles in an epithelial cell and a muscle cell of the hindgut on the seventh day of incubation (Scott *et al.*, 1984).

Dissemination of LACV in *Oc. triseriatus* kept at 25°C progressed more slowly. At 7 days, viral antigen was most abundant in midgut cells, and had started to appear in the fat body. At 9 days it was abundant in the fore- and midgut, fat body, heart and abdominal ganglia. By 14–16 days, viral antigen was abundant in the salivary glands, brain and thoracic ganglia, ovaries, oviducts and heart. The hindgut did not become infected until 16 days post-infection, and the Malpighian tubules remained uninfected for 25 days. By day 16, large amounts of antigen were detected in the pericardial cells, which is suggestive of phagocytosis (Beaty and Thompson, 1978).

In females of *Cx. tritaeniorhynchus* infected with JEV and kept at *c.* 26°C, the virus first replicated in some epithelial cells of the posterior midgut, from which it quickly spread throughout that epithelium. Specific fluorescence showed next in cells of the fat body near the midgut, and later in fat body associated with other organs. Some 10–12 days after feeding, fluorescence showed in the salivary glands, compound eyes, thoracic ganglia and Malpighian tubules. The salivary glands alone responded to fluorescent antibody staining for at least 42 days after the infective feed, so the females were thought to remain infective throughout their remaining lifespan (Doi *et al.*, 1967).

Ambient temperature affected the rates of infection and dissemination of West Nile virus in

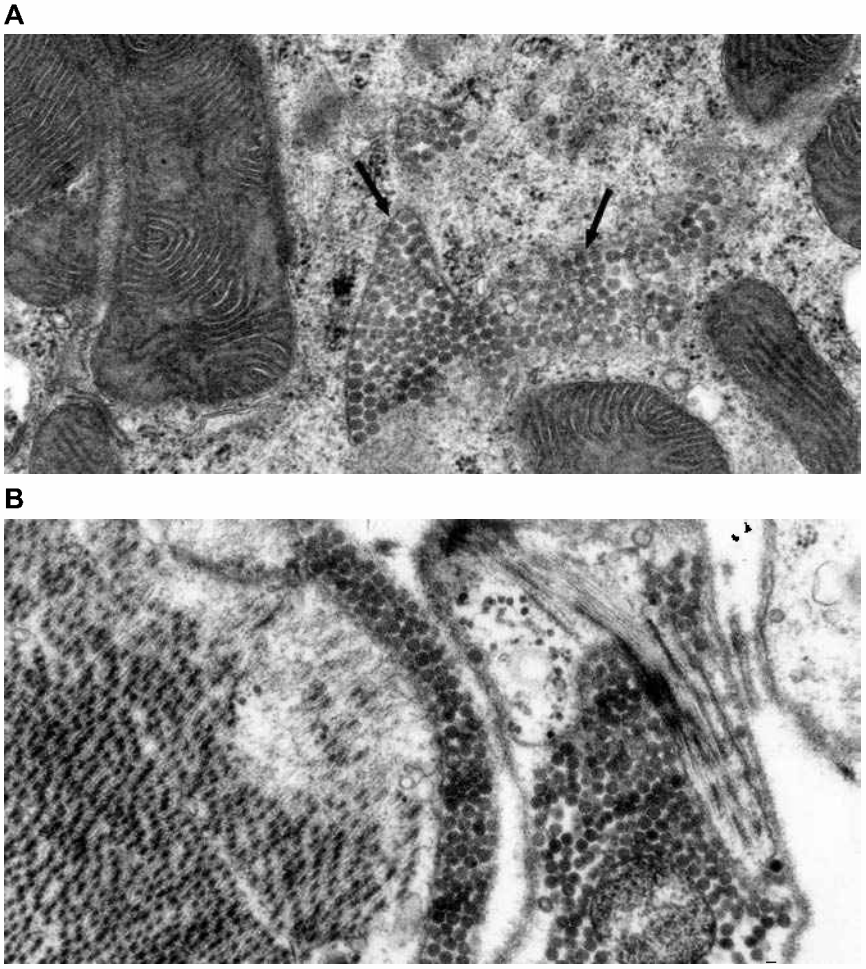

Figure 44.6 Sections through epithelial and muscle cells of the hindgut of adult female *Culiseta melanura* 7 days after oral infection with Eastern equine encephalitis virus. (Micrographs kindly provided by Dr Thomas Scott.) **A**. Part of an epithelial cell, showing aggregations of virus (denoted by arrows) suggestive of replication. Magnification × 37,000. **B**. Parts of one or more muscle fibres, showing aggregations of virus within the sarcoplasm. Magnification × 36,600.

Cx. pipiens. At 21 days post-infection, disseminated virus was found in 42% of females kept at 20°C, in 85% kept at 26°C and in100% kept at 30°C. Of females kept at 18°C, only 30% showed disseminated virus at 32 days post-infection (Dohm *et al.*, 2002a).

In most cases, virus particles disseminate from cells of the posterior midgut to the haemolymph by passing through the basal plasma membranes and traversing the basal lamina. Two studies have shown possible involvement of the cardia, a three-layered structure formed by intussusception of short regions of both foregut and midgut into the midgut lumen. Immunocytochemical examination of *Cx. pipiens* infected with RVFV showed virus in cells of all regions of the midgut. Among females that showed a midgut infection but no dissemination beyond it, the cardia was infected in only 10%, but the anterior midgut in 66% and the posterior midgut in 78%. In contrast, among females in which the infection disseminated beyond the midgut, those values were: cardia,

58%; anterior midgut, 71%; and posterior midgut, 75% (Romoser *et al.*, 1992).

An ultrastructural and immunocytochemical study of *Cx. pipiens* infected with RVFV showed virions within and between the epithelial layers of the cardia. Many were budding into the so-called matrix (an extension of the basal lamina where it has penetrated between layers of epithelium). From there, virus budded extensively through the basolateral plasma membranes and into the basal labyrinth of cells. Virus replication in the cardia preceded passage into the haemocoel (Lerdthusnee *et al.*, 1995).

Scott *et al.* (1984) suggested that the phases of infection and dissemination progress more rapidly with alphaviruses than they do with flaviviruses or bunyaviruses. The few relevant descriptions of infection and dissemination presented earlier are consistent with that suggestion.

(e) Invasion of salivary-gland cells and replication

The structure and ultrastructure of the salivary glands is described in Volume 1, Section 12.1. Briefly, contained distally within the cytoplasm of each salivary gland cell is an extracellular cavity bounded by plasma membrane. The lumen of this 'apical cavity' opens to the lumen of a lateral salivary duct. Following dissemination of virus through the haemocoel and invasion of salivary gland cells, replication commences in the cell cytoplasm.

In *Cs. melanura* infected with EEEV, sections of salivary gland fixed at 55 h and 69 h post-infection showed some salivary gland cells to be infected. Naked nucleocapsids occurred in small numbers within the rough endoplasmic reticulum, and in larger numbers on the cytoplasmic side of the plasma membrane surrounding the apical cavity. Nucleocapsids were seen at different stages of budding through the plasma membrane and entering apical cavities, within which they matured to infectious virions. Enveloped virions were present within the apical cavity – some close to the plasma membrane, others dispersed throughout the lumen (Figure 44.7). These findings suggested that *Cs. melanura* might first be able to transmit EEEV ≤3 days after feeding (Scott and Burrage, 1984).

Among females of *Cx. pipiens* that were examined daily after feeding on mice viraemic with SLEV, virus particles were first found in the salivary glands after 8 days, when only very few of the gland cells were infected, yet already at that time females were capable of transmitting the virus to mice. Enveloped particles were dispersed within cisternae of the endoplasmic reticulum, and less frequently within Golgi bodies. By day 12, the number of infected cells was greater and the number of virus particles had increased substantially. By day 19, virions were evenly dispersed throughout the extracellular cavities and were present in the lateral and common salivary ducts. Eventually, virions in the extracellular cavities assembled in dense paracrystalline arrays (Figure 44.7). At a time when salivary gland cells were heavily infected, only small numbers of virions could be seen in the cells of most other organs (Whitfield *et al.*, 1973).

(f) Invasion of ovaries and oviducts

The infection of ovaries, and particularly of germ cells, is key to the vertical transmission of arboviruses. A number of descriptions of arbovirus dissemination make no mention of infection of ovarian tissues or cells, and, in some cases where there is mention, the ovaries are among the last organs to be infected. In females of *Cx. pipiens* infected with RVFV, ovarian tissues were not infected at a time when infection was evident in associated ducts (the calyces, lateral and common oviducts) and the genital chamber (Romoser *et al.*, 1992). After the dissemination of EEEV in *Cs. melanura*, no virus was present in the ovarioles although virus was found in all other organs examined, including the lateral and common oviducts (Scott *et al.*, 1984). Some 10–12 days after infection of *Cx. tritaeniorhynchus* with JEV, specific fluorescence showed that the virus had disseminated to a number of organs. It was seen in ovariolar sheaths but not within oocytes (Doi *et al.*, 1967).

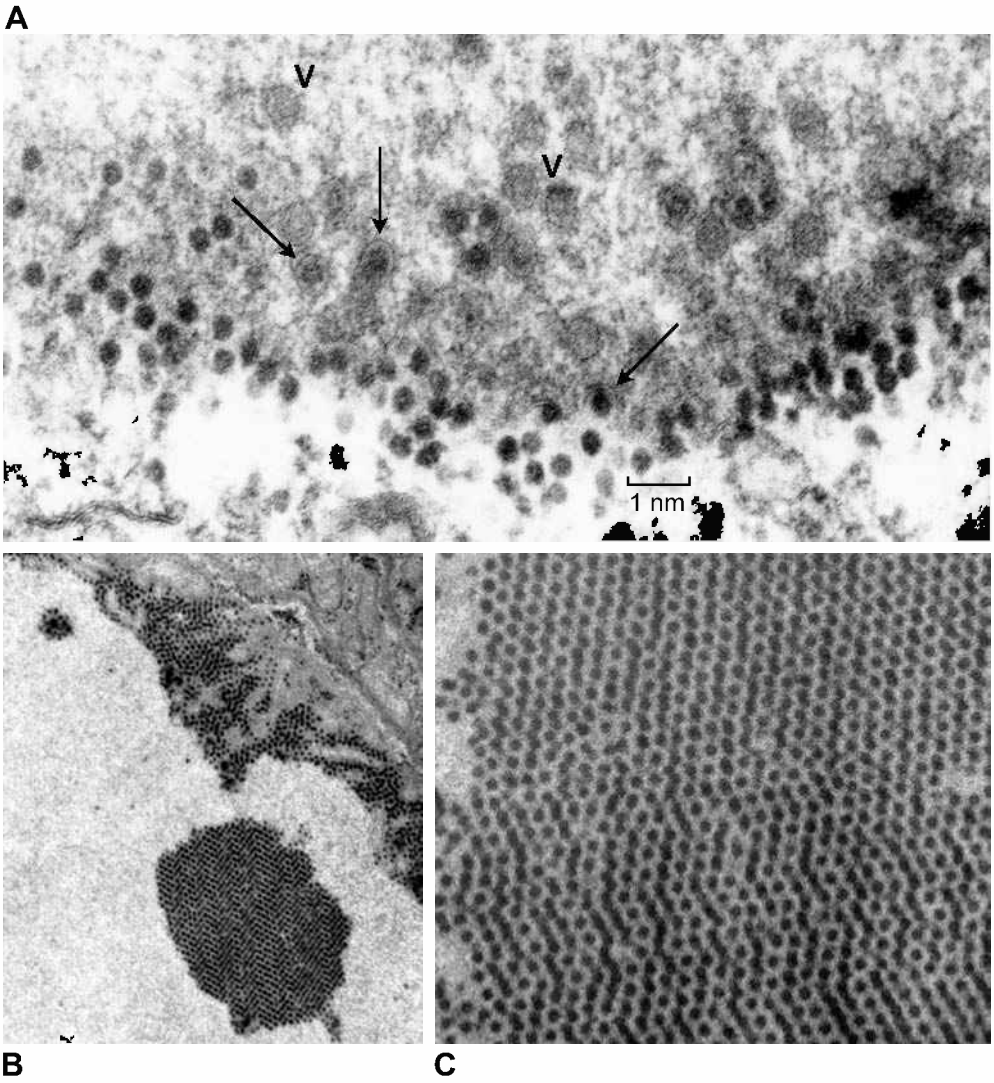

Figure 44.7 Sections through parts of salivary-gland cells of adult female mosquitoes orally infected with an arbovirus some days earlier. **A.** *Culiseta melanura* during the third day after infection with Eastern equine encephalitis virus. (Micrograph kindly provided by Dr Thomas Scott.) An indistinct plasma membrane separates the cell cytoplasm (below) from part of an apical cavity (above). Naked nucleocapsids are present on the cytoplasmic side of the plasma membrane, while enveloped virions (V) are present in the apical cavity within an amorphous secretory matrix. Arrows point to virions that appear to be in different stages of budding through the plasma membrane. **B, C.** Crystalline aggregations of St. Louis encephalitis virus in salivary gland cells of *Culex pipiens* 25 days after its infection. (Reproduced from Whitfield *et al.* (1973), with permission.) **B.** A crystalline aggregation of virus particles within the apical cavity and near its bounding plasma membrane. Magnification × 20,000. **C.** Part of a crystalline array, illustrating stacking defects and variations in appearance due to the plane of the section. If the third dimension of this crystal was similar to the two in the plane of the section, it would have contained >50,000 particles. Magnification × 71,000.

(g) Persistence

It is generally thought that, once an arbovirus has invaded a mosquito and become transmissible, the mosquito remains infective for the rest of its life. Evidence comes from at least three virus/mosquito combinations. Female *St. aegypti* transmitted dengue virus to human volunteers from 11 to 75 days after becoming infected (Siler *et al.*, 1926). Female *Cx. univittatus*, infected with West Nile virus and kept at 26°C, attained 100% transmission to chicks up to 81 days after infection (Jupp, 1974). Circumstantial evidence suggests that yellow fever virus can survive through severe dry seasons in South America (which last up to 4 months) within infected females of *Haemagogus janthinomys* (Section 44.7.4.d).

Further evidence comes from an investigation into the replication of virus in ageing females of *Oc. triseriatus* infected with LACV. Genomic RNA was detected in all midguts from days 3 to 28 post-infection (when last tested). The percentage of females with detectable cRNA (complementary RNA) declined to zero over that period, whereas the percentage in which mRNA was detectable declined only slightly and not significantly. It appeared that LACV replication declines in the midguts of persistently infected females, but that stable virions remain in these tissues for long periods, perhaps for the life of the mosquito.

Females of *Oc. triseriatus* that were given a single blood meal infective with LACV, but then no further blood meals, became gonotrophically inactive after the one ovarian cycle. At intervals between days 14 and 30 post-infection their ovaries were assayed for genomic RNA, cRNA and mRNA. The proportions of females with detectable viral RNA in their ovaries at 28 and 30 days, respectively, post-infection were: for genomic RNA, 100% and 70%; for cRNA, 30% and 10%; and for mRNA 10% and 1%. Virus replication increased in the ovaries of mosquitoes that took a non-infective blood meal at 30 days post-infection, and RNA replication was continuous in the ovarian follicles of females that took repeated meals. Whether that included the oocytes was not clear. Chandler *et al.* (1996) concluded that, as long as gonotrophic activity is maintained by blood feeding, virus replication continues.

Presence of virus within a mosquito host is not a measure of its transmissibility. Among females of *Cx. tarsalis* infected with WEEV and kept at 20, 25 or 30°C, transmission rates peaked at 7–10 days after infection, but decreased markedly during the following 10–15 days, although the virus titre within the bodies remained (Reisen *et al.*, 1993).

44.8.2 Host-cell penetration by bunyaviruses

A universal mechanism among animal cells for internalizing functionally important molecules is termed receptor-mediated endocytosis. This form of endocytosis involves the internalization of stretches of membrane into which receptors have clustered after binding to a ligand. The cytoplasmic surfaces of these stretches of membrane are coated with the protein clathrin. They invaginate, forming so-called coated pits; these bud off forming coated vesicles which contain clathrin, adaptors and receptors in the vesicle membrane and the ligand in the interior. Within the host cell, the vesicles fuse with one another to form a larger body called an endosome. In the mildly acidic interior of the endosome, viral fusion proteins undergo conformational change, exposing residues which mediate fusion between the virus envelope and the endosome membrane, leading to release of nucleocapsids into the cytoplasm. The now uncoated viral genome initiates transcription, and the replication cycle in the cell cytoplasm begins. The process of receptor-mediated endocytosis as it occurs when developing oocytes of *St. aegypti* internalize the yolk protein vitellogenin from the haemolymph is described in detail in Volume 1, Section 20.4.

(a) Receptor-mediated endocytosis of bunyaviruses

Animal viruses exploit receptor-mediated endocytosis to penetrate host cells. The first step in penetration is binding to a specific moiety on the plasma membrane, which serves as receptor.

When a bunyavirus particle comes into contact with the surface of a suitable host cell, attachment proteins in the virus envelope bind to receptor molecules in the plasma membrane. The plasma membrane invaginates at that point, forming a coated pit which buds off as a vesicle that encloses the virus particle, and then breaks away internally.

Studies of cell penetration have been undertaken with La Crosse virus and other strains of California encephalitis virus (CEV) (*Orthobunyavirus*) and also with Uukuniemi virus (UUKV) (*Phlebovirus*) which infects small mammals and for which ticks and mosquitoes serve as vectors. All arboviruses have four structural proteins, of which two are glycoproteins named Gc and Gn (Section 44.1.1.a), which function in cell penetration and which have been studied in detail in bunyaviruses. The bunyavirus virion is bounded by a bilayered lipid envelope, from which protrude many cylindrical spikes with overall lengths of 5–10 nm (Figure 44.1). The spikes contain two glycoproteins, Gc and Gn (previously called G1 and G2). A single, continuous open reading frame in the medium-sized (M) RNA segment of the genome encodes the glycoproteins, and the primary gene product is co-translationally cleaved to give mature Gc and Gn (Bupp *et al.*, 1996; Andersson *et al.*, 1997; van Regenmortel *et al.*, 2000). In UUKV, Gc and Gn are about 75 and 65 kDa, respectively. Later, Gc and Gn accumulate, together with the nucleocapsid, in the membranes of the Golgi complex, which is the budding site of the viruses. At that stage, the glycoproteins are organized into the cylindrical spikes of the envelope (Persson and Pettersson, 1991; Rönkä *et al.*, 1995).

The penetration of virus particles into cells and their intracellular transport involve interaction with receptor molecules in the plasma membrane and with cytoplasmic organelles, respectively; for these processes to proceed the glycoproteins must be in appropriate molecular conformation, e.g. as monomers, homodimers or heterodimers. The conformations of Gc and Gn have been investigated experimentally, but under *in vitro* conditions that were remote from those at the surface of or within target cells in the natural host. Experimental conditions probably explain the differences in conformation of Gc and Gn claimed by different investigators. Persson and Pettersson (1991) reported that, after processing in the endoplasmic reticulum and Golgi complex, the Gc and Gn of Uukuniemi virus are incorporated into virus particles as heterodimers. Rönkä *et al.* (1995) reported that those glycoproteins exist in native form as homodimers, not as heterodimers. They stated that Gc can exist as a pH-insensitive homodimer. In contrast, at pH 6.4 or above, Gn exists partly as a homodimer and partly as a monomer; at pH 6.0 or below it dissociates completely into a monomer.

All investigators agree that an appropriate pH is necessary for receptor binding and entry into cells. Experiments with cultured and therefore undifferentiated cells showed that a mildly acidic environment was necessary for the entry of California encephalitis virus into both mammalian and mosquito cells. It stimulated polykaryon formation within the cells and altered the conformation of Gc (Hacker and Hardy, 1997). The Gcs of LACV and UUKV played an important role in the entry of those viruses into cells, first functioning as the attachment protein, and then, by undergoing a pH-dependent conformational change, triggering fusion with the endosome membrane. The presence of Gn was essential for critical fusion sequences (Pekosz and González-Scarano, 1996; Plassmeyer *et al.*, 2005).

The pH of the blood bolus in engorged mosquitoes is known only for the first hour after its ingestion. In *Cx. pipiens*, the pH of chicken blood, normally 7.51, increased to a mean of 7.68 (range 7.47–7.90) during that period. The pH of duck blood, normally 7.65, decreased to a mean of 7.52 in *Cx. pipiens*, and increased to a mean of 7.75 in *Anopheles quadrimaculatus* (Volume 1, Section 14.1.2).

(b) Binding studies with LACV glycoproteins

The binding capacities of the envelope glycoproteins of La Crosse virus were studied by first labelling the virus with [^{35}S]-cysteine. Then further

steps, including isolation and lysing, led to the production of purified [^{35}S]-Gc and [^{35}S]-Gn. Measurements of binding capacity were undertaken with suspensions of undifferentiated cells from a mammalian cell line (E6 Vero) and a mosquito cell line (MAT, from *Oc. triseriatus*) (Ludwig *et al.*, 1991).

Gc from LACV bound to the mammalian cells in a dose-dependent manner, with the amount bound being proportional to the amount contained in the culture vessel. The efficiency of binding was approximately 70% (Figure 44.8A). In contrast, increase in the amount of Gn from LACV added to cultures did not result in a proportionate increase in the amount of Gn bound to cells, and binding efficiencies were less than 5%. Similar studies with the mosquito cells produced different results: both Gc and Gn bound to the mosquito cells in a dose-dependent manner (Figure 44.8B). The binding curves make the binding capacity of Gc appear greater than that of Gn. However, the mass of Gn is less than that of Gc; therefore, on a molar basis, the difference in binding capacity is less substantial. The binding efficiencies were close to 100% for both glycoproteins.

Incubation of labelled Gc and Gn from LACV with dissected midguts from *Oc. triseriatus* gave another binding profile. The Gn molecules from LACV bound to midgut cells in a dose-dependent manner, but the Gc molecules did not bind (Figure 44.9).

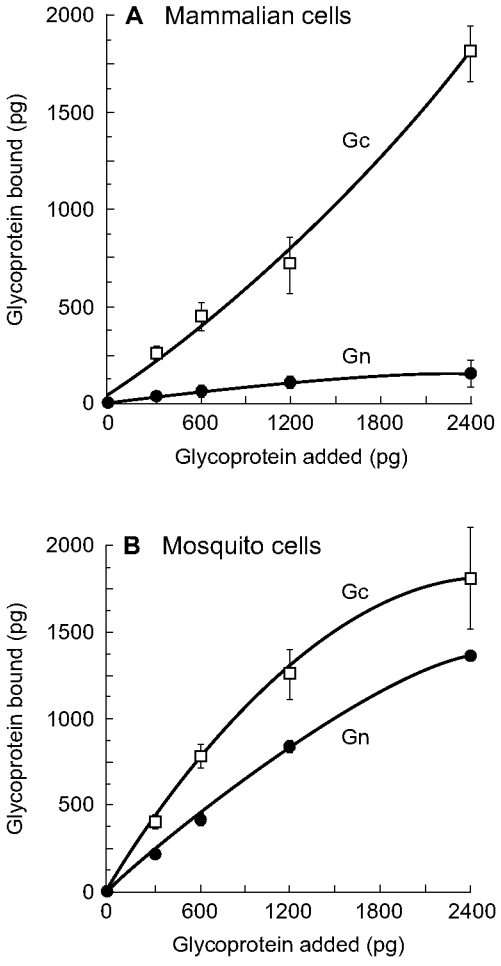

Figure 44.8 Binding curves for [^{35}S]-cysteine-labelled envelope glycoproteins (Gc and Gn) from La Crosse virus incubated with undifferentiated cells in suspension. A. Mammalian cells (E6 Vero). B. Mosquito cells (cell line MAT, from *Ochlerotatus triseriatus*). Means ± 95% confidence limits. (After Ludwig *et al.*, 1991.)

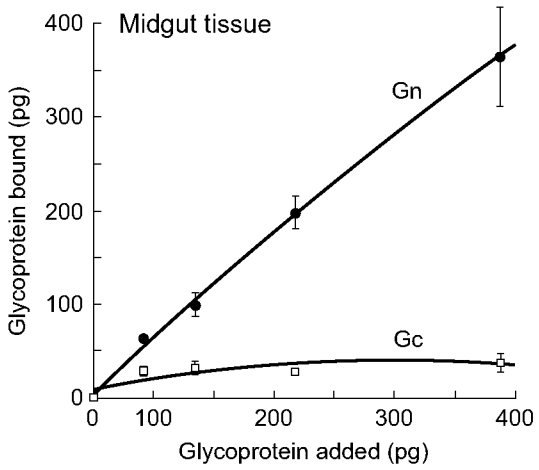

Figure 44.9 Binding curves for [^{35}S]-labelled envelope glycoproteins (Gc and Gn) from La Crosse virus incubated with midguts from adult *Ochlerotatus triseriatus*. Means ± 95% C.L. (After Ludwig *et al.*, 1991.) Midguts of adult females were removed, cut transversely, washed and incubated for 1 h at 37 °C with the labelled glycoproteins. After further washing and processing, the radioactivity of the specimens was measured and converted to pg glycoprotein.

To confirm that the binding of labelled Gc and Gn was receptor mediated, the binding studies were repeated in the presence of excess unlabelled glycoprotein. With the mammalian cells, the binding of labelled Gc was significantly reduced by excess Gc. The effect of Gc on binding of Gn was not tested because Gn did not bind anyway. With the MAT line of mosquito cells, the binding of labelled Gc and Gn was inhibited by the presence of excess homologous unlabelled glycoprotein, but not by that of excess heterologous glycoprotein. Excess heterologous protein (Gn) enhanced the binding of Gc (Figure 44.10A). The effect of excess Gc on the binding of Gn was less easy to interpret (Figure 44.10B).

To examine the ability of excess Gc and Gn to inhibit infection of cells by LACV, 25 plaque-forming units of LACV were mixed with serial dilutions of purified glycoprotein, and added to cell monolayers in the wells of tissue-culture plates. The cultures were incubated for 1 h, washed, and incubated for a longer period; finally, the virus content was measured. Addition of excess Gc (0.5 µg/well) to the initial 1 h incubation with the mammalian cells blocked infection; addition of excess Gn had no effect. In contrast, infection of the mosquito cells could not be blocked by incubation with excess Gc or Gn alone, but only by excess of both Gc and Gn (0.5 µg + 0.5 µg/well).

Ludwig *et al.* (1991) drew the following conclusions from those findings. (i) The initial event in the infection of cells by LACV is interaction of envelope glycoproteins with specific receptor sites. (ii) The Gc of LACV bound to undifferentiated mammalian and mosquito cells. The Gn bound to undifferentiated mosquito cells but not to the undifferentiated mammalian cells. Therefore, Gc is the viral attachment protein for (undifferentiated) vertebrate cells. (iii) Only Gn could bind to the isolated mosquito midguts *in vitro*; Gc could not bind. Therefore, in the differentiated cells of the midgut epithelium, only loci coding for Gn receptor sites are expressed. (iv) Because Gc bound to undifferentiated mosquito cells, expression of loci coding for Gc receptor sites in differentiated cells of some mosquito tissues other than midgut is a possibility. (v) Infection of the undifferentiated mosquito cells was prevented only by a combination of Gc and Gn; therefore, those cells possess separate receptors for Gc and Gn.

44.8.3 Barriers to dissemination

When a mosquito has imbibed virions in a blood meal, infection of the mosquito and subsequent

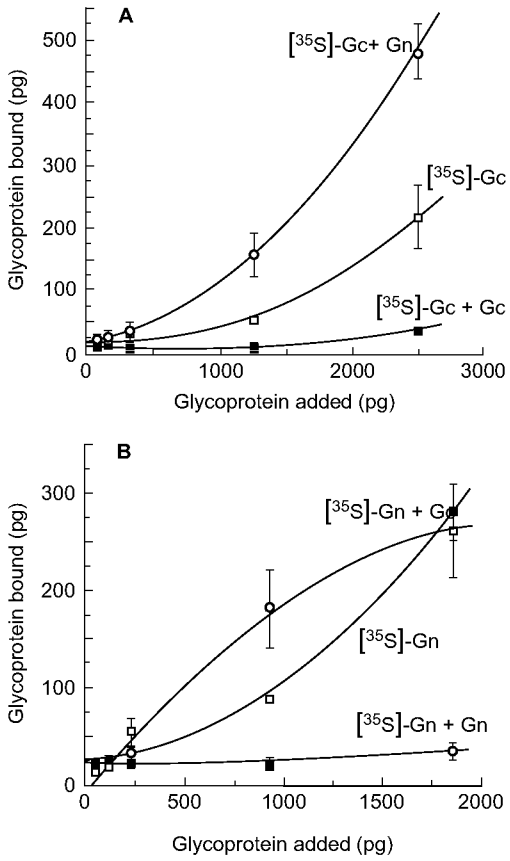

Figure 44.10 Inhibition (or enhancement) of binding of [^{35}S]-labelled envelope glycoproteins (Gc and Gn) from La Crosse virus to undifferentiated mosquito cells (cell line MAT) when incubated in the presence of excess unlabelled homologous or heterologous glycoprotein. (After Ludwig *et al.*, 1991.) **A.** Binding curves for [^{35}S]-Gc when alone or in the presence of excess Gc or Gn. **B.** Binding curves for [^{35}S]-Gn when alone or in the presence of excess Gc or Gn. Means ± 95% confidence limits.

transmission of the arbovirus require the following steps: (i) invasion of cells of the midgut epithelium by arbovirus particles; (ii) replication of virus, and movement of particles out of the midgut epithelial cells; (iii) dissemination of virus through the haemocoel or alternative conduit; (iv) entry of virus into salivary gland cells, and replication; (v) passage of virus particles into the apical space of salivary gland cells; and, finally, (vi) carriage of virus particles in saliva through the salivary ducts. Any system that blocks or fails to support virus entry into or exit from cells of the midgut epithelium or salivary glands is described as a 'barrier', the term having a broad connotation. The fact that in orally infected mosquitoes the ovaries become infected much later than some other organs, and that the oocytes become infected later than other ovarian cells, suggests the presence of one or more barriers in the ovary, the molecular nature of which is not known. The prevalence or effectiveness of a particular barrier may differ between different populations of a host species.

(a) Midgut barriers

Two types of midgut barrier to infection are distinguished: (i) midgut infection barriers, which prevent infection of cells of the midgut epithelium by virus particles within the midgut lumen; and (ii) midgut escape barriers, which prevent viruses that have penetrated into and replicated within cells of the midgut epithelium from escaping from them into the haemocoel. In practice, the absence of virus from within the midgut epithelium is taken to indicate a midgut infection barrier, whereas the presence of virus in the midgut epithelium but not elsewhere indicates a midgut escape barrier.

'Apparent' midgut infection barriers must be examined carefully because virus density in a blood meal can affect the probability of infection. Among females of *Cx. univittatus* that had fed on chicks viraemic with West Nile virus and that imbibed a titre of $10^{5.0}$ mouse LD_{50}, 100% were infected at 15 days post-feeding. In contrast, of females that had imbibed a titre of $10^{2.6}$, only 43% were infected at 16 days post-feeding (Jupp, 1974). The existence of such 'infection thresholds' has been reported for a number of virus/host combinations (Hardy *et al.*, 1983).

In an investigation to find whether Germany's most abundant floodwater mosquito, *Aedimorphus vexans*, could serve as a vector of Sindbis virus, females were infected with a local strain of Sindbis virus by either intrathoracic inoculation or feeding on infected bovine blood. Intrathoracic inoculation led to a 350-fold amplification of virus within 5–7 days, whereas females that ingested the virus proved refractory to infection. At 12 and 24 h after feeding on a blood/virus suspension, virus titres could be accounted for by the amount of infected blood remaining in the midgut lumen, indicating the presence of a midgut infection barrier (Modlmaier *et al.*, 2002).

Among females from a long-colonized strain of *Cx. pipiens* that had ingested $10^{4.2–7.2}$ PFU of RVFV, 78% developed midgut infections, while, of the females with midgut infections, a disseminated infection developed in only 33%. These results suggested the existence of midgut-infection and midgut-escape barriers. Among the females with disseminated infections, 95.6% were able to transmit virus by bite and 4.4% were unable to transmit (Turell *et al.*, 1984a). A midgut escape barrier is possibly present in *Cx. tarsalis*. In females that had developed a midgut infection with WEEV, the virus particles started to replicate but did not reach maturation, with naked nucleocapsids accumulating along the basal margins of the midgut cells and progressing no further (Hardy *et al.*, 1983).

The most extensive investigations into barriers have involved infections with La Crosse virus of females of two closely related species – *Oc. triseriatus* and *Ochlerotatus hendersoni*. Experiments with three strains of the former and seven strains of the latter showed that a midgut infection barrier was present in 2% of females of both species, and that a midgut escape barrier occurred more frequently among *Oc. triseriatus* (27%) than *Oc. hendersoni* (12%) (Table 44.9). Therefore, LACV disseminated through the haemocoel in a higher percentage of *Oc. hendersoni* (86%) than *Oc. triseriatus* (71%). Further evidence for a midgut escape barrier in *Oc. triseriatus* was

Table 44.9 Barriers to infection by La Crosse virus in two species of *Ochlerotatus*. Above. The relative occurrence of barriers to infection in adults of *Oc. hendersoni* and of *Oc. triseriatus*, and rates of transmission by those mosquitoes. Mean values for colonized mosquitoes of geographically distinct strains. (Recalculated from the data of Grimstad *et al.*, 1985.) Below. The relative occurrence of barriers to infection in batches of small, normal-sized or large females of *Oc. triseriatus* (Walton strain), and rates of transmission by those mosquitoes. (Recalculated from the data of Grimstad and Walker, 1991.)

Test batch	*Batch size* (*n*)	*Midgut infection barrier* (%)	*Midgut escape barrier* (%)	*Proportion transmitting* (%)	*Postulated salivary gland barrier* (%)	*Layers in basal lamina* (*n*)
Ochlerotatus hendersoni						
Means of 7 strains	433	2	12	7	79	–
Ochlerotatus triseriatus						
Means of 3 strains	548	2	27	59	12	–
Ochlerotatus triseriatus						
Small	21	0	0	90	10	3–6
Normal size	43	0	14	70	16	–
Large	26	0	31	41	28	9–16

Mosquitoes were infected by allowing 7- to 10-day-old females to imbibe defibrinated rabbit blood containing $10^{6.0}$–$10^{6.9}$ $TCID_{50}$ (50% tissue culture infective dose) ml^{-1} LACV from a membrane feeder, feeding *ad libitum*. After the extrinsic incubation period, individual females were allowed to feed on individual suckling mice, which were observed for 7–10 days for signs of infection. Presence or absence of virus in the midgut was tested for by plaque assays. Mosquitoes not transmitting to suckling mice were tested for viral antigen by indirect immunofluorescence of head-squash preparations. A positive head squash showed that virus had disseminated from the midgut. The proportion of each group presumed to have a salivary-gland barrier was estimated by subtracting from the total tested the numbers with midgut barriers plus the numbers transmitting.
–, Not measured.

provided by the markedly higher transmission rate in parenterally infected females compared with orally infected females. In contrast, in Oc. *hendersoni* the two transmission rates were virtually identical (Grimstad *et al.*, 1985; Paulson *et al.*, 1989; Grimstad and Walker, 1991).

Some insight into the nature of a barrier was obtained by examining LACV infections in adults of Oc. *triseriatus* of three body-size classes – small, normal and large – obtained by rearing larvae under different dietary regimes. Among orally infected females, those that had endured the severest nutritional deprivation as larvae (the small-adult class) transmitted LACV at a higher rate (82%) than females from the moderately deprived class (54%) or the non-deprived class (52%) (Grimstad and Haramis, 1984). That finding led to a search for midgut barriers in normally cultured batches of Oc. *hendersoni* and Oc. *triseriatus*, and in small, normal-sized and large adults of Oc. *triseriatus* (Walton strain). Virtually all of these mosquitoes developed a midgut infection, confirming the absence of a midgut invasion barrier (Table 44.9). No midgut escape barrier was found in the small Oc. *triseriatus*, but such a barrier was present in 31% of the large females (Grimstad and Walker, 1991).

Reports by a number of investigators that the temperature at which mosquito larvae were reared later governed the infection of otherwise susceptible females that had ingested virus might indicate an effect on midgut infection barriers. The infection of Oc. *taeniorhynchus* (not a natural host) with RVFV and VEEV provides a clear example of this phenomenon. Adult females that had passed their aquatic stages at 19°C were significantly more susceptible to infection with those viruses than females that had been reared at 26°C, regardless of whether, after feeding, they were kept at 19°C or 26°C (Turell, 1993).

(b) Salivary gland barrier

Where virus particles have disseminated beyond the midgut but do not appear within the salivary gland cells, a 'salivary-gland infection barrier' exists, and, where virus is present with the salivary gland cells but cannot be transmitted, a 'salivary-gland escape barrier' is thought to exist.

In 86% of Oc. *hendersoni*, LACV disseminated beyond the midgut, but a significantly lower percentage of infected Oc. *hendersoni* than of Oc. *triseriatus* was able to transmit LACV to mice ($p < 0.001$) (Table 44.9). Virions were present within the salivary glands of infected Oc. *hendersoni*, so its poor transmission rate was ascribed to the presence of a salivary-gland escape barrier (Grimstad *et al.*, 1985; Paulson and Grimstad, 1989). To find whether the salivary glands of infected females were impaired in some way, the ability of infected females to probe and locate blood in rabbit ears was examined, but no difference was found between infected and control females (Paulson *et al.*, 1992). It was of interest whether concurrent infection with a parasite might affect the transmission of LACV. In two experiments, among batches of Oc. *triseriatus* that were concurrently infected with *Plasmodium falciparum* and LACV, 70% and 86% of females transmitted LACV to mice, a score not significantly different from that of control batches infected only with LACV (60% and 68%). In contrast, in two batches of Oc. *hendersoni* infected only with LACV, 10% and 12% of females could transmit LACV, whereas, in batches concurrently infected with *P. falciparum* and LACV, 67% and 71% of females could transmit LACV. Paulson *et al.* (1992) could only speculate on the effects of *P. falciparum* that might promote transmission of LACV.

Among Cx. *tarsalis* orally infected with WEEV, a percentage was capable of transmitting the virus by bite, and that percentage increased from 33% to 88% as the dosage ingested increased from $10^{1.7}$ to $10^{6.1}$ PFU/sample. Dissection of organs from the non-transmitters showed that in some females viral antigen was present in the midgut but not other tissues, indicating a midgut escape barrier. Among the other non-transmitters, viral antigen was present in quantity outside the midgut, where it was absent from the salivary glands and found mostly in 'remnant tissues' from the dissection. This was taken to indicate a salivary-gland infection barrier. The effectiveness of the midgut-

escape and salivary-gland infection barriers was determined partly by the quantity of virus imbibed and partly by the ambient temperature. At 18°C temperature was dominant, and in most females a midgut escape barrier blocked dissemination of the virus. At 26°C, the effectiveness of both barriers was lost at a high infective dose. At 25°C and 32°C the midgut escape barrier was less restrictive, but the salivary-gland infection barrier was effective. Exposure to 32°C for more than 6 days was detrimental to transmission. For longer holding periods, 25°C was the most favourable temperature for transmission (Kramer *et al.*, 1981, 1983).

(c) Ultrastructural studies

All internal organs of insects are surrounded by a basal lamina, which forms a barrier to movement of macromolecules into or from the haemolymph. Such movements are governed by both the porosity and charge of the lamina (Reddy and Locke, 1990). The basal lamina surrounding the ovarian follicles of *St. aegypti* is freely permeable to molecules or particles that have dimensions <11 nm (Anderson and Spielman, 1971). Particles of 5–8 nm were the largest to consistently permeate the basal lamina surrounding the midgut of *Cx. tarsalis* (Houk *et al.*, 1981). The charge on the various particles was not known. On this evidence, the basal laminae of mosquito species might be expected to block or slow the passage of viruses from the midgut into the haemolymph, and equally from the haemolymph into other organs. Characteristic virion diameters are: for bunyaviruses, 80–120 nm; for flaviviruses, 40–60 nm; and for togaviruses, 70 nm (Sections 44.1.1.a, 44.1.2.a and 44.1.5a).

Basal laminae vary in thickness and structure. In large females of the *Oc. triseriatus*, the basal lamina surrounding the midgut consisted of 9–16 layers and had a mean thickness of 0.24 μm, whereas in small females it consisted of only 3–6 layers and had a mean thickness of 0.14 μm. Because a midgut escape barrier to La Crosse virus was present in 31% of large females but was absent from all small females (Table 44.9), Grimstad and Walker (1991) postulated that thinner basal laminae permit a more rapid dissemination of LACV into the haemocoel. The part of the alimentary canal called the cardia (or oesophageal invagination) is a possible site of entry of arbovirus particles. In *Cx. pipiens*, the cardia contains a region called the 'matrix', which has a spongy texture and appears to be modified basal lamina (Section 44.8.1.d). In females infected with RVFV, putative virions were present in large numbers in the matrix, and extensive budding of virions from cells of the cardia directly into the matrix was observed (Lerdthusnee *et al.*, 1995). In *Oc. taeniorhynchus*, the basal lamina had a spongy texture at locations where it was situated between midgut cells and overlying muscle fibres (Romoser *et al.*, 2004).

Particles of Venezuelan equine encephalitis virus that expressed green fluorescent protein (GFP) in infected cells were used to trace the distribution of VEEV in *Oc. taeniorhynchus* and, in particular, to compare the distributions in orally infected and inoculated females. The modified virus was able to infect cells once and to express GFP, but it was incapable of replication, so the GFP revealed the first cells and tissues to be infected. In orally infected females, cells of the midgut epithelium were the only cells infected. In inoculated females, cells in a number of organs became infected, but not those of the midgut epithelium. Cells of the tracheae and visceral muscles were consistently infected, and to a greater extent than those of other tissues. Because genetically unmodified virus inoculated into the haemocoel could infect cells of the midgut epithelium, whereas genetically modified virus could not, it was argued that normal virus in the haemocoel must infect the complex of tracheae and visceral muscles before it infects midgut epithelial cells (Romoser *et al.*, 2004).

(d) Genetics

As in other bunyaviruses, the genome of LACV is segmented into three RNA molecules, designated large (L), medium (M) and small (S). When a mosquito becomes infected with two closely related viruses, reassortment, i.e. exchange of segments

between members of the two viruses, produces reassortant viruses (Section 44.1.1.c). In one study, reassortants of two strains of California encephalitis virus, namely LACV and snowshoe hare virus (SSHV), which had exchanged M segments, were compared for their ability to infect *Oc. triseriatus*, a natural host of LACV. The two reassortants were similar in their ability to infect midgut cells: i.e. 98% infection when they contained the M segment of LACV, and 92% when they contained the M segment of SSHV. However, reassortants containing the M segment of SSHV disseminated from the midgut to infect other organs in only 26% of cases, whereas reassortants containing the M segment of LACV disseminated in 96% of cases. Both SSHV and a reassortant containing the M-segment RNA from SSHV were inefficiently transmitted by bite to mice; in contrast, both LACV and a reassortant containing the M-segment RNA from LACV were efficiently transmitted (Beaty *et al.*, 1981, 1982). The investigators concluded that the medium-sized RNA segment of LACV is the major determinant for dissemination of LACV from infected midgut cells of *Oc. triseriatus*, and that it may be a major determinant for transmission to vertebrate hosts.

Differences in prevalence of the different barriers to dissemination of LACV were found between strains of *Oc. hendersoni* and between strains of *Oc. triseriatus*. For example, marked differences were shown between two strains of *Oc. triseriatus* (Uken, Kentucky (F_4) and Underc, Michigan (F_5)) in prevalence of the midgut-infection and escape barriers and the supposed salivary-gland barrier. These were the result of genetic differences, not dietary manipulation, because the females had developed from well-nourished larvae (Grimstad *et al.*, 1985; Grimstad and Walker, 1991).

Wild populations of a mosquito species may differ in susceptibility to infection by a particular arbovirus. Laboratory measurements were made of the susceptibility of 28 geographic strains of *St. aegypti* to oral infection with a long-passaged strain of yellow fever virus. Twenty-one of the mosquito strains were tested when in the F_0–F_3 generations from the wild. In brief, the East African and Central and South American domestic populations were more susceptible to infection than the West African sylvan populations (Tabachnick *et al.*, 1985). Comparison of two populations of *St. aegypti* for susceptibility to oral infection with YFV showed a colony of *St. a. formosus* from Ogbomosho in Nigeria to be somewhat less susceptible than a colony of *St. a. aegypti* from Puerto Rico. The virus seldom disseminated beyond the gut in Ogbomosho females, indicating the presence of a midgut escape barrier, but it disseminated through the body to the salivary glands in females from Puerto Rico. Crosses between the two strains yielded F_1 progeny with intermediate susceptibility, implying codominance. The results of backcrosses indicated a single major controlling locus and modifying minor loci. Despite the low susceptibility of the Ogbomosho population to infection with YFV, a major epidemic of yellow fever occurred there in 1987 (Section 45.3.5.c).

Three other flaviviruses (dengue viruses 1–4, Uganda S virus and Zika virus) showed the same pattern of dissemination in strains of *St. aegypti* as that described for YFV above, i.e. they disseminated at low to high rates in females from Puerto Rico, but not at all in females from Ogbomosho (Miller and Mitchell, 1991). Studies on the genetics of midgut-infection and midgut-escape barriers for dengue virus in *St. aegypti* are described in Section 45.2.5.b.

44.8.4 Extrinsic incubation period

Usually, the time between ingestion of virus-infected blood by a mosquito and attainment of capacity to transmit the virus to a susceptible host (the extrinsic incubation period, EIP) shortens with rise in temperature. Thus, for *Oc. triseriatus* infected with EEEV, the extrinsic incubation period for 50% of females was 16 days at 21°C, 11 days at 27°C, and 4–5 days at 32°C (Chamberlain and Sudia, 1955).

With *Cx. tarsalis*, the estimated temperature thresholds for zero transmission of WEEV and SLEV were 10.9°C and 14.9°C, respectively. Above those thresholds the number of degree days

required for median transmission was 67.6 for WEEV and 115.2 for SLEV. Degree-day models were used to calculate monthly changes in the EIP for WEEV and SLEV in the San Joaquin and Coachella Valleys, California, based on 'mosquito temperatures', which were estimated by combining nocturnal air temperatures and diurnal resting-site temperatures. Temperatures were, on average, 5°C lower in the San Joaquin Valley than in the Coachella Valley, and the potential transmission seasons in the two valleys were calculated to be, respectively, 10 and 8 months for WEEV, and 8 and 5 months for SLEV (Reisen *et al.*, 1993).

A rather different result was obtained with *Oc. vigilax* infected with Ross River virus, in which virus replication and dissemination occurs rapidly and the EIP is short. At temperatures of 18, 25 and 32°C, virus was first detected in the salivary glands on days 3, 2 and 3 post-infection, respectively. Based on the extent of dissemination, and the known effective absence of a salivary gland escape barrier, the EIPs for those three temperatures were estimated to be 5, 4 and 3 days, respectively. Kay and Jennings (2002) concluded that, in the field, ambient temperature has little effect on Ross River fever epidemiology.

To find whether the EIP is modified by the condition of constant temperature under which mosquito colonies are usually kept, batches of *Aedimorphus fowleri* infected with RVFV were kept at a constant 17°C, a constant 28°C, or under a regime that cycled between 17°C and 28°C each 24 h to mimic the temperatures to which a mosquito might be exposed during October in south-east Senegal. The time between the viraemic blood meal and first transmission by bite was 35 days at constant 17°C, 18 days at cycling 17°C and 28°C, and 35 days at constant 28°C. The EIP under the cycling temperature was similar to that at a constant 26°C (Turell, 1989).

In a different approach to the same problem, a cohort of *Haemagogus spegazzinii* (cited as *Haemagogus capricornii*) that had fed on monkeys viraemic with YFV was divided into batches that were kept at a constant 20, 25, 30 or 35°C, and exposed daily to suckling mice. The date of first transmission (shortest EIP) was determined for each temperature. At a constant 20°C or 35°C virus replication was not completed, but exposure to 35°C for short periods was tolerated. The shortest EIP was longer at 25°C than at 30°C. The EIP was significantly shortened when the ambient temperature was raised from 25°C to either 30°C or 35°C for 4 h each day (Table 44.10). Species of *Haemagogus* are sun-loving insects, and are exposed to sunlight in open areas of the high canopy and in open patches in forest where some individuals bite when at ground level. The 20:4 h, 25:35°C regime was thought to resemble the natural conditions more closely than a constant 25°C or 30°C (Bates and Roca-García, 1945a).

EIP is affected by the amount of virus that is imbibed. When females of *Hg. spegazzinii* fed on monkeys with low, moderate or high-titred viraemias of YFV, and were kept at a constant 30°C, shortest EIPs of >18, 13 and 10 days, respectively, were measured (Table 44.10). When YFV was

Table 44.10 Effects of ambient temperature, either constant or varying, and of virus titre, on the extrinsic incubation period (EIP) of yellow fever virus in *Haemagogus spegazzinii*, which became infected by feeding on monkeys with measured viraemias. (From the data of Bates and Roca-García, 1946b.)

Temperature (°C)	*Viraemia titre*	*Shortest EIP (days)*	*Total transmissions* (n)
20	High	–	0
25	High	28	4
30	High	10	13
35	High	–	0
25:30	High	23	14
25:35	High	12	12
30	High	10	
30	Moderate	13	
30	Low	>18	

After taking an infective blood meal, batches of 44 females were kept at one of three constant temperatures, or each day were kept at 25°C for 20 h and at either 30°C or 35°C for 4 h. The mean number of days to first transmission of YFV to infant mice was recorded for each batch of mosquitoes. For comparison of constant and variable temperatures, the total number of transmissions was also recorded.

imbibed in low titre, characteristic of viraemic capuchins (*Cebus*), few or no *Hg. spegazzinii* became infected. In contrast, when it was imbibed in moderate titres, characteristic of infective squirrel monkeys (*Saimiri*), most became infected, and when imbibed in high titres, characteristic of infective night monkeys (*Aotus*), almost all became infected (Bates and Roca-García, 1945a, 1946b).

In a study in Bangkok, the F_2 adult female progeny of wild-caught *St. aegypti* ingested blood from rhesus monkeys infected with a twice-passaged local strain of DENV-2. The mosquitoes were kept at a number of constant temperatures between 20°C and 35°C, and their ability to transmit the virus to monkeys was tested at 3–7-day intervals for a total of 25 days. After feeding on a monkey with a viraemia of $10^{3.3}$ PFU ml^{-1} plasma, females that were kept at 26°C could not transmit the virus, even within 25 days, but the females kept at 30–35°C could transmit it. The EIP was 12 days for mosquitoes kept at 30°C, and 7 days for females kept at 32°C or 35°C (Watts *et al.*, 1987).

The EIPs of 7–12 days for DENV-2 in *St. aegypti* measured in Bangkok should be considered in relation to the lifespan of *St. aegypti*. Estimates of the mean daily survival rate of *St. aegypti* in Bangkok, made over the period of one year and for both sexes combined, gave $p = 0.88$, corresponding to a life expectancy of 8.5 days (Sheppard *et al.*, 1969).

44.9 PATHOLOGY OF INFECTED MOSQUITOES

A number of laboratory studies have shown that oral infection of mosquitoes with an arbovirus can reduce female fecundity and shorten adult female lifespan. The fecundity of female *Cs. melanura* infected with EEEV was significantly reduced compared with that of uninfected controls, and their mean survival rate was reduced, but not until some 14–21 days post-infection. But, because the probability of females taking a further blood meal and transmitting virus was highest 7–10 days after becoming infected, infection with EEEV should not affect the probability of its further transmission (Scott and Lorenz, 1998). In contrast, the mean duration of survival of females of *Coquillettidia perturbans* with disseminated infections of EEEV was markedly reduced from as early as day 8 post-infection onwards (Moncayo *et al.*, 2000b).

When *Cx. pipiens* of Egyptian origin fed on guinea pigs that were either uninfected or viraemic with Rift Valley fever virus, females in which disseminated infections developed produced significantly fewer eggs than uninfected females. This might be explainable, in part, by competition for metabolic resources. Females with disseminated infections were significantly less successful in completing a second blood meal, and survived for a shorter period than uninfected females (Turell *et al.*, 1985; Faran *et al.*, 1987). RVFV is often transmitted mechanically between its vertebrate hosts, when virulence in its mosquito vectors would be irrelevant.

Females of *Culex tarsalis* that imbibed defibrinated blood containing WEEV at titres of 2.7–3.0 log $TCID_{50}$/female survived as well through 21 days as the uninfected controls. Females that imbibed the higher dose of 4.7–5.0 log $TCID_{50}$ showed increased mortality from day 12 post-infection onwards, and by day 18 post-infection 99% had died, compared with 80.5% of the controls. These females also spent less time in spontaneous flight than the controls (Lee *et al.*, 2000).

Pharate first-instar larvae within post-diapause eggs of *Oc. triseriatus* suffered significantly higher cumulative mortality if vertically infected with La Crosse virus than if uninfected (McGaw *et al.*, 1998) (Section 45.5.6.b). Vertical infection of *Oc. dorsalis* and *Oc. melanimon* with California encephalitis virus did not significantly affect larval survival but significantly lengthened the mean duration of the larval stage, an effect which, in the field, could be deleterious (Turell *et al.*, 1982c).

Studies of cell or tissue damage in adult female mosquitoes orally infected with arbovirus have revealed deleterious effects on the midgut epithelium and salivary glands. In the case of *Cs. melanura* infected with EEEV, sections through the

midgut cut 2–5 days after oral infection showed many infected cells; the cytology of some was normal, but others were degenerating. The studies also showed that infected cells had sloughed off into the midgut lumen. At some locations where cells had sloughed, the basal lamina had lost its integrity, and virus within the cytoplasmic debris of the degenerating cells was in direct contact with the haemocoel (Weaver *et al.*, 1988). Infection of a strain of *Cx. tarsalis* that was highly susceptible to WEEV caused necrosis and sloughing of epithelial cells; such effects were not seen after intake of WEEV by females of a less susceptible strain. Weaver *et al.* (1992) suggested that the sloughing of infected cells into the midgut lumen may ameliorate the effects of infection.

Arboviruses replicate greatly within cells of the salivary glands of their mosquito vectors, and virions accumulate in huge numbers in the extracellular cavities. Light microscopy of *St. aegypti* orally infected with Semliki Forest virus showed a gross depletion of cytoplasm from the salivary glands, apparent disorganization of cell structure, and changes in cell membranes. In almost all sections some small 'islands' of cytologically normal secretory cells could be found. Changes were first seen 1 week after infection, and were maximal at 3 weeks, when the mosquitoes had difficulty in blood feeding and transmission rates were low (Mims *et al.*, 1966).

Tissues of *Cx. quinquefasciatus* infected with WNV were collected on days 3, 7, 14 and 21 post-infection for examination by electron microscopy. On day 3, structural changes were apparent in the endoplasmic reticulum of the midgut cells, and virus particles visible in the basal lamina surrounding the midgut were taken to be escaping into the haemocoel. In midgut samples taken on days 7 and 14, increasing numbers of epithelial cells showed signs of virus replication and structural change. On day 21, intensive replication was apparent in midgut muscle cells (Girard *et al.*, 2005).

The salivary glands were first infected with WNV 5–8 days after first infection of the individual. WNV replication was apparent by day 11 post-infection, and by day 14 all salivary gland cells were infected and some contained paracrystalline masses of virions. At that time, some salivary gland cells showed cellular degeneration and signs of apoptosis. The percentage of salivary glands containing apoptotic cells increased from 14% at 14 days to 71% at 21 days and 83% at 28 days post-infection, whereas the frequency never exceeded 33% in uninfected salivary glands. Although the mean titre of WNV in saliva did not change significantly over time, the percentage of saliva samples containing WNV decreased. Cell death frequently occurred in salivary glands examined at 21 and 28 days post-infection, equating to approximately 4 days of viral replication in the glands (Girard *et al.*, 2005, 2007).

These laboratory investigations showed a significant reduction of remaining lifespan in some but not all infected mosquito hosts, in certain cases resulting in an apparent reduction in virus transmission. Cytopathological effects on the midgut epithelium that lead to apoptosis of damaged cells might be protective or might cause fatal damage, while apoptosis of salivary gland cells could diminish production of the saliva that is important for blood feeding. However, the findings from experiments with colonized mosquitoes and cultured arboviruses can be misleading. It is known that, where in nature populations of a mammalian host and a pathogen occur together, the interactions between them generate selective forces that affect the virulence of the pathogen and the refractoriness of the host, with the possibility over time of modification of either. In other words, there is a trade-off between virulence and refractoriness (Sections 42.1.2, 43.4.3.b). It seems probable that in the field comparable trade-offs develop between arboviruses and their mosquito vectors.

44.10 PATHOLOGY OF INFECTED VERTEBRATES

44.10.1 Introduction

The effects of arboviruses on vertebrates range from inapparent infections in some amplifying hosts to

fatal effects in others, and often produce fatal effects in dead-end hosts. Infections in amplifying hosts lead to high-titred viraemias that last for a number of days, but generally the animals do not show clinical effects or suffer severe morbidity. In contrast, infections in dead-end hosts produce viraemias of relatively low titre at most, cytopathology is a common consequence of replication in invaded cells, and infected animals often suffer severe morbidity and high rates of mortality. Vertebrates that survive infection with an arbovirus become immune to it for the rest of their lives.

Humans and domesticated animals are known to be dead-end hosts of a number of arboviruses. Relatively little evidence has been reported of wild vertebrate species suffering severe effects from arboviral infections, and where it is known it is uncertain how well these species fit into the categories of amplifying or dead-end hosts. Certain genera of South American monkeys infected with YFV show severe morbidity and suffer a high mortality rate. Strains of YFV differ in virulence, so the severity of the disease can vary with viral strain. Wild birds are the amplifying hosts of West Nile virus, and many bird species tolerate infection with WNV; however, mortality has been reported from some species, possibly where WNV has invaded a new geographical region or where it has increased in virulence.

Almost all arboviral infections of humans are zoonoses – the viruses having a maintenance cycle in wild animals and infecting humans as dead-end (tangential) hosts (Figures 45.3, 45.34). O'nyong-nyong fever may be an exception, as humans are its only known vertebrate host. With dengue virus and yellow fever virus, the importance of zoonotic transmission has been reduced in historical time owing to environmental and other changes, and transmission cycles involving only the virus, mosquitoes and humans have become established.

Arboviruses tend to be most virulent in dead-end hosts, and the pathogenesis of arboviral infections has been most intensively investigated in humans and domesticated animals. The following pathological conditions are associated with different arboviral infections: (i) invasion of central nervous system cells resulting in aseptic meningitis, encephalitis (inflammation of the brain) or meningoencephalomyelitis (inflammation of the meninges and of the brain and spinal cord); (ii) undifferentiated fever; (iii) febrile, systemic illness with invasion of the cells of visceral organs, causing haemorrhage, cardiovascular instability, and varying degrees of hepatic and renal insufficiency; (iv) arthritis, with inflammation of joints; and (v) abortion. On the basis of their pathological effects, arboviral diseases are classified as encephalitides or haemorrhagic diseases. Examples of both are described below. Definitions of some medical terms can be found in the Glossary (Appendix 3).

44.10.2 Encephalitides

The encephalitides are viral infections of the central nervous system (CNS). Encephalitis relates to any inflammation of the brain; encephalomyelitis relates to any inflammation involving both brain and spinal cord. In dead-end hosts, all encephalitides produce long-lasting neurological sequelae in some of the survivors. Arboviruses that cause encephalitis in humans or domesticated animals are found in four families: *Flaviviridae* (flaviviruses, e.g. JEV, MVEV (Murray Valley encephalitis virus) and WNV); *Togaviridae* (alphaviruses, e.g. EEEV, VEEV, WEEV); *Bunyaviridae* (orthobunyaviruses, e.g. CEV); *Reoviridae* (coltiviruses, e.g. Colorado tick fever virus).

(a) Japanese encephalitis

Individual arboviruses that cause encephalitis may infect hosts of more than one vertebrate class, e.g. both birds and mammals. Japanese encephalitis virus is an example. Birds, and notably waterbirds, are amplifying hosts of JEV; among these, ardeids are known to produce substantial viraemias. Farmed pigs develop substantial viraemias when infected, and are the principal amplifying host. They show little other evidence of infection, but infection of sows can lead to fetal abortion. Almost all other domesticated animals are susceptible to infection, as evidenced by seroconversion, but few show clinical

symptoms. Horses infected with JEV may develop encephalitis, when they suffer a high fatality rate.

Japanese encephalitis (JE) is the most common cause of viral encephalitis in the 'Asian–Pacific' Region, and can be found from the extreme south-east part of the Russian Federation to Papua New Guinea and the extreme north of Australia, and from Japan to the west of India. The disease is endemic in parts of China, and in eastern, southern and south-eastern Asia. Some 50,000 cases of JE occur annually, with 25% to 35% case fatality rates, and more than 30% severe long-term disabilities in survivors. In fact, the estimate of 50,000 cases of illness a year is probably an underestimate, because of inadequate surveillance and reporting, and because most infections are asymptomatic. In rural villages, exposure and infection occur at a very early age, with half of all cases occurring in children less than 4 years of age. Typical incidence rates in those younger than 19 years ranges from 10 to 100 per 100,000 population per year (WHO, 2011).

In parts of Asia where mass immunization of children against JE is not practised, by early adulthood nearly all surviving individuals have protective antibodies. In a cohort of primary-school children in Tamil Nadu (India), where JE was endemic, the overall incidence of clinical JE was 15 per 10,000 children aged 5–9 years, while the proportions that seroconverted in two successive years were 0.38 and 0.42. Among the serologically positive children, only about 1:270 developed clinical symptoms (Gajanana *et al.*, 1995b).

The pathogenesis of JE has been reviewed by Innis (1995b), Tsai *et al.* (1999), Solomon *et al.* (2000) and Solomon and Vaughn (2002). From observations on laboratory mice, it is thought that after inoculation by an infective mosquito the virus first replicates outside the CNS, causing a small, transient viraemia which enables it to invade the CNS. JEV has been isolated from human blood, brain and cerebrospinal fluid, but only exceedingly rarely from other human tissues. However, when complete autopsies were carried out, histopathological lesions were found chiefly in the CNS but also in a number of other organs, suggesting that virus replication was not limited to the CNS. In humans, clinical symptoms range from a mild febrile illness to severe meningoencephalomyelitis, with infected neurons scattered through the CNS. The neuropathological findings readily explain the clinical expression of the disease. Convulsions occur in many young patients, and some form of motor paralysis commonly occurs. Among clinically positive cases the death rate is approximately 35%, and 30–50% of survivors suffer grave neurological sequelae.

Over a period of 5.5 years, 132 children admitted to a hospital in Lucknow, India, were diagnosed as suffering acute JE. Of these, 40 died in hospital and another two died after release from hospital. Owing to the demand for beds, patients were discharged as soon as they could feed. At the time of release, 32 of the 90 long-term survivors were in a state of altered consciousness, such that they made only a slight response to verbal commands and did not recognize family members. Attempts were made to contact all survivors, and 55 were seen and assessed on at least one occasion 12–18 months after the initial illness. Among those 55 patients, 16 had no neurological abnormality, 14 suffered minor neurological sequelae (such as scholastic backwardness and subtle neurological symptoms) and 25 suffered major neurological sequelae (Table 44.11). Examination of 22 patients at more than 2 years and of seven at more than 3 years after release showed further improvement in certain conditions in some patients. Upper motor neuron types of weakness were not detectable in four of seven patients who had shown them earlier, but cerebellar symptoms and lower motor neuron types of weakness persisted in all who had shown them (Kumar *et al.*, 1993).

Mass immunization of children has greatly reduced the incidence of JE in Japan, South Korea, China and some other countries. Interestingly, the seroprevalence of vaccine-induced neutralizing antibodies declines with age. Despite these advances, JE is now the most important cause of epidemic encephalitis in humans worldwide (Tsai *et al.*, 1999; Solomon and Vaughn, 2002).

Table 44.11 Survival rate and neurological sequelae among 132 children aged 6 months to 12 years diagnosed with acute Japanese encephalitis in Lucknow, India. (From the data of Kumar *et al.*, 1993.) Attempts to contact all survivors resulted in the clinical assessment of 55 of the former patients at 12–18 months after the initial illness. The true proportion of cases with sequelae might have been lower than that shown because patients recovering well were less likely to report for follow-up examination.

Details of patients	*Number of cases*	*Proportion of total cases (%)*	*Proportion of follow-up cases (%)*
Total patients with acute JE	132	100	–
Died	42	31.8	–
Survived	90	68.2	–
Survivors seen at 12–18 months	55	41.7	100
No neurological sequelae	16	–	29.1
Minor neurological sequelae	14	–	25.4
Major neurological sequelae	25	–	45.5
Frank motor deficits	18	–	32.7
Frank mental retardation	12	–	21.8
Convulsions	10	–	18.2

(b) California serogroup encephalitides

Specific antibodies to a number of strains of California encephalitis virus are found in humans, but not all of those strains produce serious clinical effects. A handful of clinical cases have been ascribed to infection with California encephalitis virus itself. Jamestown Canyon virus often produces a respiratory infection in addition to neurological illness, mostly in adults. Snowshoe hare virus has been implicated in rare but clinically severe neurological infections in the USA and Canada (McJunkin *et al.*, 1998, review). La Crosse virus, the most severely pathological member of the group, is the principal cause of encephalitis in North America. Infections with Inkoo virus or Tahyna virus had the same clinical manifestations – fever or neuroinfection or both. Neuroinfection took the form of aseptic meningitis, meningoencephalitis or encephalitis. All manifestations led to chronic neurological disease in a percentage of patients (Demikhov, 1995; Demikhov and Chaitsev, 1995).

Infections with La Crosse virus in sciurid rodents, its amplifying hosts, cause viraemia but no obvious clinical manifestations. In contrast, the laboratory mouse, which has been used as an animal model, is seriously affected by infection with LACV; one PFU administered peripherally to young mice is fatal. Following subcutaneous injection into suckling mice, replication in adjacent muscle causes a systemic viraemia. A high virus titre in blood is a determinant of neuroinvasion, which may lead to CNS infection, encephalitis and death. LACV replicates within brain neurons. Peripheral virulence and neuroinvasiveness co-segregate with the medium-sized RNA segment (Borucki *et al.*, 2002, review).

LACV continues to be a major cause of encephalitis and aseptic meningitis in children in most states east of or contiguous with the Mississippi River. The incidence of the disease in endemic areas is estimated at 10–30 cases per 100,000 population, only some 3% of infections being in persons of 20 years or older. Approximately 50% of patients hospitalized with the disease have seizures; encephalography (EEG) showed abnormalities in more than 90% of patients tested. The fatality rate in hospitalized patients is 1%, and about 25% of the survivors suffer recurrences (McJunkin *et al.*, 1998, review). Most clinical cases of La Crosse encephalitis are in children. In a study of 127

patients hospitalized in Charleston, West Virginia, from 1987 to 1996, most were in the age range 6 months to 15 years. Serious effects of infection included seizures, aseptic meningitis, increased cerebral pressure and cerebral herniation. All of the patients survived, but at discharge 12% showed neurological deficits which, 10–18 months later, had increased in severity (McJunkin *et al.*, 2001).

(c) *Eastern equine encephalitis*

Horses and other equines are very susceptible to infection with Eastern, Western and Venezuelan equine encephalitis viruses (*Alphavirus*, family Togaviridae). The equine encephalitides occur only in the Americas. In addition to the hosts considered below, Eastern equine encephalitis (EEE) causes high rates of mortality in pigs and in farmed birds of several species – turkey, chicken, pheasant and emu (Weaver, 2001).

Birds. Wild birds of at least three orders are amplifying hosts of EEEV. In Louisiana, a number of ciconiiform and passerine species gave positive results to serological tests. Viraemias of about 48 h duration developed in white ibis (*Eudocimus albus*) and snowy egrets (*Egretta thula*) inoculated with low doses of EEEV. The birds did not appear unwell during the viraemias, and survived well. Smaller birds infected in the laboratory (northern cardinal, *Cardinalis cardinalis*; red-winged blackbird, *Agelaius phoeniceus*; house sparrow, *Passer domesticus*) all showed very high viraemia; the infections were fulminating and mostly ended with death within 48 h (Kissling *et al.*, 1954b).

In Connecticut, EEEV was isolated from a wild turkey (*Meleagris gallopavo*) and from crows, including two individuals identified to species as American crows (*Corvus brachyrhyncos*). Examination of their brains revealed a pathology characteristic of viral encephalitis. In the case of the wild turkey, this included perivascular accumulations of lymphocytes, a reduction in macrophages, scattered foci of gliosis and necrosis, and also, in some other organs, evidence of haemorrhage (Beckwith *et al.*, 2002).

There have been no reports of high death rates among wild birds at foci where epornithic transmission was known to be occurring, but post-mortem examination of small numbers of birds that were dead when found showed infection with EEEV (Howard *et al.*, 1996). The survival of wild adult blue jays (*Cyanocitta cristata*) that were naturally seropositive for EEEV-specific antibodies was significantly lower than that of seronegative birds, as indicated by the reappearance rates of ringed birds one year later. In contrast, infection seemed not to affect survival of adult or juvenile Florida scrub jays (*Aphelocoma coerulescens*) (Garvin *et al.*, 2004).

Horses. Sporadic epidemics of Eastern equine encephalitis in North America involve up to tens of thousands of equines and dozens of humans. In equines, the symptoms of infection with EEEV (Lineage I) include blindness, anorexia, involuntary movement of the head, photophobia, somnolence and prostration. Some 80–90% of horses infected with EEEV die and, among the survivors, many suffer severe neurological sequelae. An outbreak in north-east Mexico in 1996, involving Lineage I EEEV, was thought to have resulted from introduction of the virus by migratory birds. Within an area with a population of 862 horses, 113 horses displayed symptoms consistent with EEE, and 91% of the affected animals died (Brault *et al.*, 1999).

In Central and South America, where EEEV occurs as Lineages II–IV (Section 45.1.1), it is less virulent. Equine outbreaks are common in some regions, occasionally causing death, but there is little evidence of human disease (Scott and Weaver, 1989; Weaver, 2001).

Humans. In North America, most infections in humans are inapparent or subclinical, but when there are clinical manifestations the effects can be serious. We examine three surveys of clinical cases from different periods: two from Massachusetts and the third from 11 states. Correlations between age and clinical findings varied between the three surveys, but whether this was due to the relatively small case numbers, to demographic differences or to changes with time over a period of decades is not apparent. Feemster (1957) surveyed 50 clinical

cases from Massachusetts for the years 1938, 1955 and 1956, with the following results: age <2 years, 21 cases (42%); age 2–9 years, 13 cases (26%); age 10–60+ years, 16 cases (32%). Death rates were: 0–9 years, 64.7%; 10–60+ years, 75%. Brain damage led to sequelae in almost 70% of survivors, these being most severe in children under 5 years of age. Przelomski *et al.* (1988) surveyed 16 clinical cases from Massachusetts for the period 1970–1984: age 1–9 years, five cases (31.2%) with one death (20%); age 10–49 years, five cases (31.2%) with no deaths; age 50–69 years, six cases (37.5%) with four deaths (66.7%). Deresiewicz *et al.* (1997) surveyed 36 clinical cases from 11 states for the period 1988–1994, but reported only summarily on correlations with age. The age range was 6 months to 85 years, and the median age 59 years; 36% of the patients died, and no correlation between death rate and age was found. Among the survivors, 21.7% suffered severe, 13% moderate and 60.8% mild sequelae.

Among the clinical cases reviewed by Deresiewicz *et al.* (1997), the illness usually began with a period of up to 28 days of apparent benign viral illness, with fever, headache and abdominal distress. Once neurological symptoms appeared, the condition of the patients deteriorated rapidly, a high proportion becoming stuporous or comatose. CT and MRI scans revealed early manifestations of encephalitis in the form of focal lesions in the basal ganglia, thalami and brainstem. The presence of large radiographic lesions did not predict a poor outcome. Gross inspection of the brains of patients who died early in the course of the disease revealed oedema, vascular congestion of the leptomeninges (arachnoid plus pia mater), haemorrhage and encephalomalacia (softening of a part of the brain). Cortical atrophy predominated in the brains of patients who died later.

(d) West Nile virus disease

Birds. Infection with WNV is fatal to some bird species but not to others. Following the arrival of the virulent strain NY99 in New York in 1999, when hundreds of birds died, an extensive pathological examination of native and exotic birds (of eight orders and 14 species) with confirmed WNV infection was undertaken. The disease was investigated most intensively in free-ranging wild American crows, among which its incidence was particularly high. A relatively small proportion of the birds examined had encephalitis with perivascular cuffing (the cuffs composed of two to five layers of lymphocytes and plasma cells). In severely affected birds, lesions were present in the cerebrum, thalamus, optic lobe, cerebellum, medulla and cervical spinal cord, but most birds appeared to have died from multi-system disorders. Generally, virus was present in most organs, but the corvids did not exhibit the extensive infection of the brain seen in other birds, particularly that in the cerebellum. Haemorrhage was widespread: within the calvaria (vault of the skull), meninges, heart, spleen, intestine, pancreas and lungs. Other pathological conditions included myocardial and hepatic lesions, gastrointestinal necrosis and nephritis. Some crows had subacute to chronic inflammation of the adrenal gland (Steele *et al.*, 2000). Elevated viraemias developed during a period of acute infection and before death in a number of passeriform birds, including American crows, western scrub jays, common ravens, house sparrows and house finches (Komar *et al.*, 2003; Reisen *et al.*, 2005).

Squirrels. Of twelve uninfected fox squirrels (*Sciurus niger*) wild caught in Iowa, 11 were infected with WNV by mosquito bite or inoculation, and kept for up to 34 days post-exposure. One served as control. On days 2–9 post-exposure, WNV was recovered by swab from the oral and rectal cavities; it was recovered also from urine. The most common microscopic lesions in infected squirrels, present in 55%, were mild-to-moderate encephalomyelitis with perivascular cuffing and gliosis, and mild-to-severe multifocal lymphoplasmacytic interstitial nephritis, present in 64%. One of the infected squirrels developed signs of incoordination, tremors and head tilt (Platt *et al.*, 2008).

Horses. WNV is an important pathogen of horses, capable of inducing significant neurological disease. The most common expressions of infection

are a low-titre viraemia, production of anti-WNV antibodies, and subclinical illness. The appearance of more substantial clinical symptoms heralds the onset of serious disease with a death rate of >40% (Cantile *et al.*, 2001). During enzootics in New York State in 1999 and 2000, many equine cases had acute onset. Moderate-to-severe ataxia, apparent as an unsteady gait, and failure of the brain to regulate posture or the strength and direction of limb movements, occurred in most cases. Muscle fasciculation (spontaneous contraction of muscle fibres) was common. Falling, with or without the ability to rise, occurred in 23% to 50% of cases. Death rates in the 2 years were 34.8% and 42.1%; horses that survived eventually recovered fully (Trock *et al.*, 2001).

To compare the pathological and immunohistochemical characteristics of horses that succumbed during WNV enzootics in Italy and the USA, ten horses were examined during postmortem examinations, with emphasis on tissue lesions and the distribution of WNV antigen in the nervous system and major organs. Six of the horses were obtained from Tuscany in 1998, and four from New Jersey in 2000. All ten horses had shown mild-to-severe polioencephalomyelitis. In the American horses, perivascular lesions extended from the basal nuclei through the brainstem and to the sacral spinal cord, whereas the Italian horses had moderate-to-severe lesions in the thoracolumbar spinal cord and only mild rhombencephalic (hindbrain) lesions. WNV antigen was scant and identified only within a few neurons, glial cells and macrophages (Cantile *et al.*, 2001). A retrospective investigation of WNV-infected horses from the same area of Tuscany started at the end of September 2008. A mild-to-moderate polioencephalomyelitis was again found, with consistent involvement of the ventral horns of the thoracic and lumbar spinal cord, and of the lower brainstem. WNV was recovered from the cerebellum and spinal cord of some horses. An isolate from one of these Italian horses, PaAn981 [AF205883], showed >99% similarity in nucleotide sequences to WNV isolates responsible for epidemics in Romania in 1996 (RO97-50) and in Kenya in 1998 (KN3829); it also showed 98.6% similarity with isolates from a dead goose in Israel (WN-Israel 1998) and a captive bird in New York (NY99-flamingo) (Autorino *et al.*, 2002) (cf. Table 45.30).

Humans. Infection with WNV can lead to a range of outcomes, very approximately in the proportions: 100 asymptomatic : 30 febrile : 1 neuroinvasive. The symptomatic illness develops as West Nile fever, which usually is self-limited. It has an incubation period of 2 to14 days, followed by fever, excessive sweating, swollen nymph nodes and joint pains. Most symptoms disappear within 10 days, but the lymphadenopathy can last much longer. Viral penetration of the blood–brain barrier leads to direct invasion of neurons, particularly those in the brainstem and anterior horn of the spinal cord. The consequences of neuroinvasion are varied, and include loss of muscle tone (acute flaccid paralysis), inflammation of the meninges (West Nile meningitis), inflammation of the brain (West Nile encephalitis), and inflammation of the brain and its meninges (West Nile meningoencephalitis). These may lead to coma and death (Hayes *et al.*, 2005b; DeBiasi and Tyler, 2006; Kramer *et al.*, 2007).

44.10.3 Viral haemorrhagic fevers

Four physiological processes may be involved in the pathogenesis of viral haemorrhagic fevers. (i) Vascular damage. Increased vascular permeability allows leakage of plasma into tissues, leading to two problems – low blood pressure and oedema. (ii) Disorders of coagulation. The haemorrhagic manifestations may be minor, e.g. bleeding from gums and nose, or petechiae (small spots of discoloration in skin or mucous membrane that arise from an extravasation of blood). They also may be more serious, e.g. gastrointestinal bleeding. (iii) Immunological impairment, which allows uncontrolled viral replication. (iv) End-organ damage. Most often this results from viral cytopathology, e.g. hepatic damage in yellow fever.

Viral haemorrhagic fevers are caused by viruses of four families, including two with arboviruses. Among the mosquito-borne viruses, species of *Flavivirus* (family Flaviviridae) cause yellow fever

and dengue, and a species of *Phlebovirus* (family Bunyaviridae) causes Rift Valley fever (Solomon, 2003).

(a) Yellow fever

Many primates are susceptible to infection with yellow fever virus. Infections cause viraemia and they can be viscerotrophic, i.e. virus replicates in and damages organs such as the liver, spleen, heart and kidneys. The pathogenesis of simian yellow fever (YF) infections has been studied most intensively in the rhesus monkey (*Macaca mulatta*; Cercopithecidae), which ranges from China to Afghanistan and is not naturally exposed to YFV. When inoculated with YFV, rhesus monkeys develop a fulminating illness, lasting only 3–4 days, characterized by hepatic dysfunction, renal failure, coagulopathy and shock. The virus replicates and virus particles accumulate in the cytoplasm of liver cells, where the mitochondria show an early and progressive degeneration. Necrosis becomes extensive in the liver, and death soon follows (Bearcroft, 1962a,b; Monath *et al.*, 1981).

In African monkeys, some of which are natural hosts, an intense viraemia may develop, but other clinical symptoms are absent or mild (Section 45.3.2.b). In vervet monkeys (*Chlorocebus aethiops*) and patas monkeys (*Erythrocebus patas*) (both Cercopithecidae) that had been inoculated with YFV, the virus replicated and accumulated within liver cells, but only rarely caused necrosis. The principal effects on hepatocytes were dilatation of endoplasmic reticulum, a massive increase in the number of mitochondria by budding, and enlargement of mitochondria. This was associated with the production of moderately large amounts of virus, but rarely progressed to necrosis (Bearcroft, 1962a,b).

Among New World monkeys, susceptibility to infection with YFV and virulence vary in different genera. Infections are often fatal in howler monkeys (*Alouatta*) and night monkeys (*Aotus*), seldom fatal in spider monkeys (*Ateles*), and rarely if ever fatal in capuchin monkeys (*Cebus*) (Section 45.3.6.c). Infection of *Aotus trivirgatus* with a local strain of YFV by mosquito bite produced acute fatal infections, characterized by fever and a very high-titred viraemia. Autopsies revealed stomach haemorrhage and liver lesions (Bates and Roca-García, 1945b).

In humans, yellow fever can vary from a mild, anicteric, febrile illness to hepatorenal failure and death. Without good cause for suspicion, it may remain undifferentiated from some other febrile illnesses; for example, the three symptoms – fever, icterus and haematuria – apply equally to the diagnosis of leptospirosis. Needle biopsy of the liver is a safe procedure for early diagnosis of YF. Injury to hepatocytes is characterized by eosinophilic degeneration with condensed nuclear chromatin (Councilman's bodies), a distinguishing feature of infections with YFV. Yellow fever is a serious haemorrhagic infection, and it can progress, variably, through fever, liver dysfunction, renal failure, haemorrhage and circulatory collapse to death within 5 or 6 days. Degeneration of liver cells causes the jaundice that gives yellow fever its name (Monath *et al.*, 1981; Poon-King *et al.*, 1991).

(b) Dengue

Dengue is primarily an epidemic disease of urban and peri-urban settings. Some 2.5 billion people are at risk of dengue, resulting in an estimated 50–100 million infections each year. The disease is endemic in more than 100 countries, and its spread is attributed to expanding geographical distribution of the four dengue serotypes and their mosquito vectors, and particularly to the increase in urban mosquito populations (WHO, 2009). The severe form, dengue haemorrhagic fever, first occurred in epidemic form in South-East Asia in the mid-1950s, and it is now among the leading causes of childhood death in that region (Innis, 1995a; WHO, 1997).

The virulence of dengue viruses in their wild simian hosts is little known, but field observations suggest that it is not high (Section 45.2.6.b). Monkeys infected by bite in the laboratory undergo a short period of viraemia (Watts *et al.*, 1987). The strains of DENV-2 that infect monkeys

in sylvatic cycles in West Africa appear to be benign in humans (Section 45.2.7). In humans, the manifestations of dengue range from a benign, self-limited disease to one that can be fatal. Three forms have been recognized – dengue fever, dengue haemorrhagic fever and dengue shock syndrome. The term **dengue** refers to the entire spectrum of dengue viral disease. To enable physicians to select appropriate treatments for dengue patients, the World Health Organization (WHO) defined the criteria needed to distinguish the different manifestations of dengue, based on the findings of studies in Thailand in the 1950s and 1960s (WHO, 1997).

Dengue fever (DF) is characterized by high temperature, headache, myalgia, arthralgia and abdominal pain (Figure 44.11), and is self-limiting, terminating through the normal course of events. Allowing infected *St. aegypti* to feed on eight healthy volunteers, and recording the time of onset of clinical symptoms, gave a mean intrinsic incubation period of 6 days (range 4.25–8.25 days). Some individuals were infective to blood-feeding mosquitoes from 6 to 18 h before the onset of clinical symptoms (Siler *et al.*, 1926). The illness first becomes apparent as an abrupt onset of high fever, pain in joints and muscles, often retro-orbital pain, and enlargement of lymph nodes. The period of viraemia is normally 4–5 days. Not uncommonly, haemorrhage occurs in the skin, gums, nose or intestinal tract. The name 'breakbone fever', which was once widely used, reflected the severity of the joint and muscle pain. Most infections pass without symptoms or cause only mild fever (Gubler *et al.*, 1981; Solomon and Mallewa, 2001).

The severe forms of dengue, which occur in 250,000 to 500,000 patients a year, result from the increasing endemicity and co-circulations of the different serotypes of dengue virus. Infection with any of the four DENV serotypes can develop into more serious conditions – dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). **Dengue haemorrhagic fever** was said to be characterized by the simultaneous occurrence of the four major clinical manifestations: high fever, haemorrhagic manifestations, thrombocytopenia

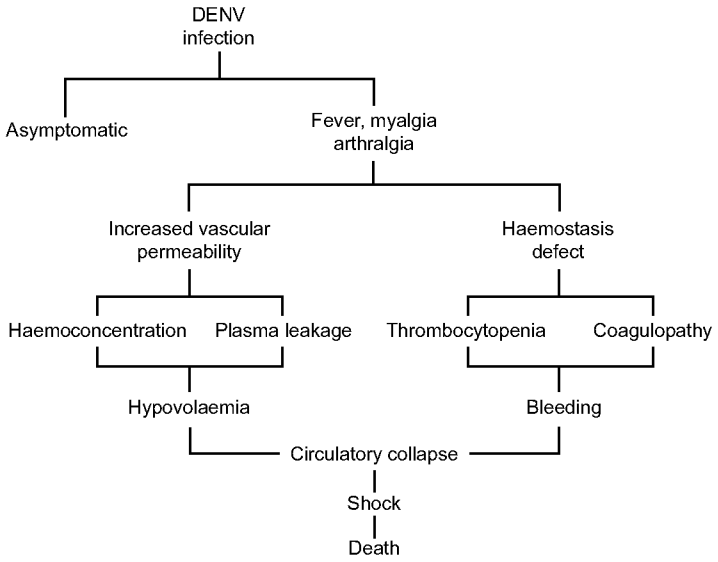

Figure 44.11 Dengue disease in humans. Sequences of pathogenesis in individuals infected with dengue virus, as dengue fever develops to dengue haemorrhagic fever and to dengue shock syndrome. (After WHO, 1997, with modifications from Deubel and Murgue, 2001.) Coagulopathy, any disorder of blood coagulation. Hypovolaemia, an abnormal reduction in the volume of circulating blood. Thrombocytopenia, a smaller than normal number of platelets per unit volume of blood.

and haemoconcentration. If infection leads to a substantial decrease in volume of circulating blood (hypovolaemia) and to serious bleeding, the condition becomes **dengue shock syndrome**, when circulatory failure can result in shock and death. In Thailand, DHF occurred more often in children than adults, and the children who suffered from DHF fell into two groups. (i) Children born to mothers positive for dengue virus antibody who when less than 1 year old developed DHF during a primary infection. (ii) Children more than 1 year-old who experienced a secondary infection with a dengue virus of a different serotype from the one that caused the primary infection; they formed about 90% of all DHF cases. Based on these and other observations from Thailand, in 1994 WHO published 'dengue clinical guidelines' for distinguishing DF, DHF and DSS, to be adopted as the standard for diagnosis, clinical management and reporting. It was not long before these guidelines were criticized, especially with regard to their use in case classification and case management by health staff under field conditions, triggering a call for their re-evaluation and standardization (Santamaria *et al.*, 2009).

DHF was thought to result from two main pathophysiological changes. (i) An increased permeability of blood vessels. This causes leakage of plasma from the blood stream into serous spaces, with consequent elevated haematocrit and low pulse pressure, and, if plasma loss becomes critical, signs of shock. (ii) Defective haemostasis. This involves thrombocytopenia (a precipitous fall in number of circulating platelets due to decreased production or decreased survival) and coagulopathy (a diminished efficiency of blood coagulation). Haemorrhage occurs in a number of organs; in order of frequency of occurrence these are the skin and subcutaneous tissue, the mucosa of the gastrointestinal tract, and the heart and liver. At autopsy, all patients who have died of DHF show some degree of haemorrhage (George and Lum, 1997; Kurane and Ennis, 1997; Rothman, 1997; WHO, 1997).

Infection of an individual with any single flavivirus, including any DENV serotype, provides long-lasting immunity against only that virus, but sequential infections with any other flavivirus or DENV serotype can occur. The immune response to exposure to a DENV serotype varies according to the individual's history of exposure to flaviviruses. In humans not previously infected by any flavivirus, an infection with a particular DENV serotype is a 'primary infection', in which anti-dengue antibodies are produced slowly and in low titre, and lose specificity over time. For at least 3 weeks after infection, the ratio of anti-dengue IgM to IgG is high. In humans previously sensitized to a DENV serotype (or another flavivirus), an infection by a different serotype is a 'secondary infection'. This is characterized by an enhanced anti-flavivirus response of the immune system elicited by epitopes common to the infecting virus and the virus responsible for the earlier sensitization. In secondary infections, antibody titres increase rapidly, the antibodies can react with many distinct flaviviruses, and the ratio of anti-dengue IgM to IgG is low (Innis, 1995a).

Working with mononuclear peripheral leucocytes from non-immune humans and monkeys that were non-permissive to DENV-2, Halstead and O'Rourke (1977) found that the leucocytes became permissive when non-neutralizing dengue antibody was added to the medium. After further experimentation, they ascribed the severity of secondary DENV infections to enhancing antibodies acquired during a primary infection, which leads to an increase of infected cells and an increase in viraemia during secondary infections. This proposed mechanism was later termed antibody-dependent enhancement (ADE). During subsequent decades ADE was one of several hypotheses proposed for the aetiology of DHF. Two very different hypotheses developed much later are described briefly here.

Noisakran *et al.* (2009) added DENV-2 to highly enriched preparations of platelets from the peripheral blood of healthy humans, and showed that dengue proteins, including NS1 and E antigens, were present on or within DENV-2 infected platelets. It was postulated that platelets may shield dengue virus from the host's immune

system, because they cannot generate the interferon-related products which control DENV replication. Entry into platelets may therefore permit replication and distribute virions throughout the host's body.

Noting that antibodies to the structural precursor-membrane (prM) of DENV form a major component of the response to infection with the virus, Dejnirattisai *et al.* (2010) proposed that the partial cleavage of prM from the viral surface reduces the density of antigen available for neutralization, leaving dengue viruses susceptible to ADE by prM-specific antibody. The combination of partial cleavage of prM, combined with cross-reaction between serotypes, makes the anti-prM response particularly susceptible to enhancement, and could be interpreted as immune evasion or even as an immune enhancement strategy of the virus.

(c) Rift Valley fever

Rift Valley fever virus induces epidemics of Rift Valley fever (RVF) in domesticated ruminants, predominantly among very young sheep, goats and cattle, in which fatality rates are high. Older animals are more tolerant of infection, but infection of pregnant females often causes abortion. Endemic African breeds are more tolerant than introduced European breeds.

The pathogenesis of RVF in ruminants is characterized by necrotic hepatitis and a haemorrhagic state; disease severity is inversely proportional to age. The disease is most acute among newborn lambs, which die within a few hours, with death rates reaching 90% or more. Fever, vomiting, incoordination, and death within a day or two may occur in older lambs and occasionally in adult sheep. In experimentally infected calves, death rates could reach 70%. In older sheep, goats and cattle, infections are often inapparent but some individuals develop acute disease. Usually, viraemia is demonstrable at the onset of fever and may persist for up to a week. Maximum titres have been reported for lambs of $10^{10.1}$, for kids of $10^{8.2}$ and for calves of $10^{7.5}$ MIP (mouse intraperitoneal) LD_{50} ml^{-1}. The clinical symptoms that follow viraemia include secretory discharges, haemorrhages and icterus. The liver appears to be the principal site of virus replication, and initial cellular changes quickly progress to a massive necrosis. As the disease progresses in neonates, the necrotic foci may enlarge to 2 mm diameter. In parts of West Africa where sheep and goats predominated numerically over cattle, the proportion of animals with RVFV-specific IgG was similar among the three species. With pregnant females, abortion is the usual, if not invariable, outcome of infection, and many may die (Daubney and Hudson, 1931; Digoutte and Peters, 1989; Ksiazek *et al.*, 1989; Morrill and McClain, 1996; Swanepoel, 1998; Boden, 2001).

For several decades after the description of Rift Valley fever in 1931, when outbreaks of the disease were limited to Africa south of the Sahara, reported human cases of RVF were uncomplicated and only very rarely fatal. Probably, some outbreaks of RVF were not diagnosed as such; the clinical characteristics of haemorrhagic fever with hepatitis are shared with other haemorrhagic fevers, including yellow fever. The epidemic in South Africa in 1975 with a small number of human deaths, and more importantly the widespread epidemic in Egypt in 1977 with many thousands of human cases and 598 recorded deaths, provided opportunities to investigate uncomplicated and complicated RVF in humans. Milder cases of RVF were characterized by fever, severe myalgia, retro-orbital pain and anorexia, lasted for 4–7 days, and mostly ended with full recovery. Some other patients were markedly icteric, and showed signs of haemorrhage such as haematemesis (vomiting of blood), melena (passage of stools stained with altered blood) and petechial skin lesions. Others exhibited meningoencephalomyelitis. Some patients suffered ocular sequelae in the form of vascular retinitis with diminished central visual acuity. Post-mortem examinations revealed profuse gastrointestinal haemorrhage and, in the liver, diffusely distributed foci of necrosis of parenchymal cells with associated haemorrhage (Van Velden *et al.*, 1977; Laughlin *et al.*, 1979).

During and after an epidemic in southern Mauritania in 1987, 348 of 600 clinically observed

patients were shown by virological and serological evidence to have had Rift Valley fever. Five major clinical manifestations of the disease were identified: mild illness, 42.8% of the cases; icteric, 28.5%; icterohaemorrhagic, 18.4%; haemorrhagic, 4.0%; and neurological, 6.3%. The mild form, common to many arboviruses, was associated with fever, pain in different body regions and weakness. The icteric form combined the characteristics of the mild illness with icterus. Together, the mild and icteric forms constituted 71.3% of the total, and had a favourable prognosis (Jouan *et al.*, 1989b). Of 18 acutely ill patients with confirmed RVF admitted to the Wad Medani Hospital (Sudan) during September to November 2007, the clinical findings, in certain patients multiple, were hepatic failure (6), acute renal failure (4), haemorrhagic manifestations (5), blindness (6), and meningoencephalitis (2). Eight patients died (Adam *et al.*, 2010).

Human death rates from Rift Valley fever vary from year to year, but, in an outline report in 2011, WHO estimated the rate to be tens of thousands. A case fatality rate of 11% in Yemen in 2000 was thought to be an overestimate due to under-reporting of mild cases of the disease (WHO, 2000b).

45

Arboviruses – case studies of transmission

All arboviral diseases of humans are or originated as zoonoses, and knowledge of their enzootiology is often necessary for an understanding of their human epidemiology. This chapter contains descriptions of the biology of seven mosquito-borne arboviruses, which were chosen to illustrate the variety and complexities of arbovirus transmission cycles. Emphasis is placed on their interactions with their mosquito vectors and vertebrate hosts.

Eastern equine encephalitis virus (EEEV) is a species of the genus *Alphavirus* and family *Togaviridae*. It shares similarities with two other species of the genus, *Western equine encephalitis virus* (WEEV) and *Venezuelan equine encephalitis virus* (VEEV). All are New World viruses, with differing but overlapping distributions. The amplifying hosts of EEEV and WEEV are birds; those of VEEV are rodents. For all three viruses, equines and humans are dead-end hosts, which suffer pathological effects that include encephalitis.

Two of the seven arboviruses described are species of the family *Bunyaviridae*. *La Cross virus* (LACV) is a strain of *California encephalitis virus*, a member of the genus *Orthobunyavirus*, while *Rift Valley fever virus* (RVFV) belongs to the genus *Phlebovirus*. LACV occurs in eastern parts of the USA, whereas RVFV is essentially African but has dispersed to parts of the Middle East. The amplifying hosts of both are wild mammals. Humans are dead-end hosts of LACV, whereas RVFV is a serious pathogen of domesticated animals, notably sheep and cattle, with high death rates among lambs and calves, and abortion among pregnant adult females.

Four of the selected arboviruses are species of the large genus *Flavivirus*, family *Flaviviridae*, viz. *Dengue virus* (DENV), *Japanese encephalitis virus* (JEV), *Yellow fever virus* (YFV), and *West Nile virus* (WNV). DENV and JEV originated in the Oriental Region and YFV and WNV in the Afrotropical Region; all have disseminated widely. The original amplifying hosts of DENV and YFV were primates; those of JEV were birds. JEV and WNV are among a clade of flaviviruses that are transmitted by species of *Culex* and that cause encephalitis in humans, which are dead-end hosts, whereas YFV and DENV are among the clade transmitted by aedine mosquitoes and cause haemorrhagic disease in humans.

45.1 TRANSMISSION OF EASTERN EQUINE ENCEPHALITIS VIRUS

In the Western hemisphere, Eastern equine encephalitis (EEE) is an infection of wild birds, and an occasional disease of imported birds, horses and humans. It was recognized as a disease long before *Eastern equine encephalitis virus* (EEEV) (genus *Alphavirus*, family *Togaviridae*) was isolated in 1933 from the brain of a fatally infected horse, and its name reflects the nature of the disease. In North America, EEEV is among the most deadly of the mosquito-borne arboviruses; the estimated case fatality rate reaches 90% in horses and approaches 80% in humans (Adams *et al.*, 2008). In this section we examine the characteristics of EEEV, the biological features of its natural transmission cycles, which involve birds and mosquitoes, and the ways in which EEEV passes from those cycles to infect horses, humans and other dead-end hosts. Because important details of the transmission cycles of EEEV are still unclear, it is uncertain how far strict definitions of the terms 'enzootic', 'epizootic' and 'epornithic' apply to those cycles (see Glossary, Appendix 3). At most EEEV foci in North America, transmission essentially involves *Culiseta melanura* as vector, and birds that live in or near fresh water or wooded swamps; such cycles are here described as enzootic. Under certain conditions, other mosquito species transmit EEEV more widely and to other birds, producing epornithic cycles. A very few species are considered to be putative vectors, transmitting EEEV to dead-end hosts.

Other information on EEEV is reported in Chapter 44, namely its replication and multiplication in mosquitoes (Section 44.8.1.e), the cytopathology observed in infected mosquitoes (Section 44.9), and the pathology of infections in birds, horses and humans (Section 44.10.2.c).

Despite some deficiencies in our knowledge, Eastern equine encephalitis virus provides a valuable model of the epizootiology/epidemiology of a mosquito-borne virus that infects wild birds, and that can be transmitted also to horses and humans with serious consequences. Further research on EEEV in the USA is likely to be limited because it has been designated a 'high consequence select agent', which imposes tight restrictions on its possession, use or transfer (*Public Health Security and Bioterrorism Preparedness Response Act*, 2002).

45.1.1 Genetic diversity

A phylogenetic tree of all known alphavirus species but one, generated from partial E1 envelope glycoprotein gene sequences, grouped the species of *Alphavirus* in four major clades. One of the four clades was divided into two subsidiary clades, of which one consisted of four EEEV lineages (I–IV) (Fauquet *et al.*, 2005).

Serological tests separated isolates of EEEV into a North American antigen type (from Canada, eastern USA and islands of the Caribbean) and a Central and South American antigen type (from Panama to northern Argentina). Nucleotide sequencing and phylogenetic analyses showed that the North American isolates constitute a single, highly conserved lineage (lineage I), with subgroups that differ in length of isolation and, to some extent, in location. The Central and South American isolates showed additional genetic diversity that resulted in three distinct lineages (II–IV), of which two (II and III) were widespread and one (IV) was represented by a single isolate from eastern Brazil (Figure 45.1). By PRNT (plaque

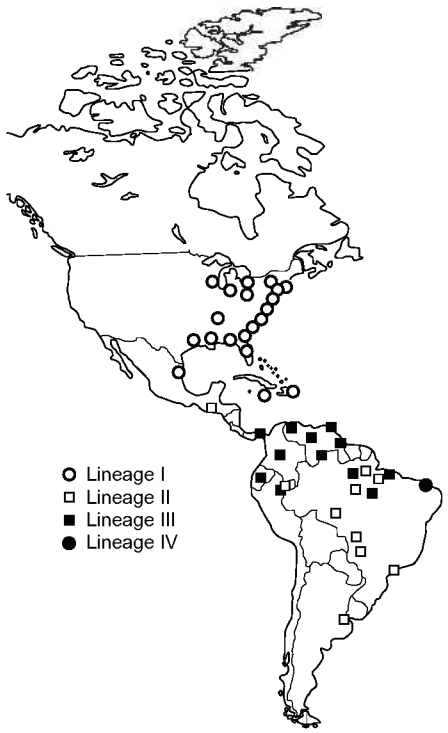

Figure 45.1 Map showing the sources of isolates of the four lineages of Eastern equine encephalitis virus in North, Central and South America. (After Brault *et al.*, 1999; with added data from O'Guinn *et al.*, 2004.)

reduction neutralization test) it was shown that each of the four lineages represents a distinct antigenic subtype (Brault *et al.*, 1999). Similar findings were obtained by Arrigo *et al.* (2010), who sequenced and analysed the structural-protein ORFs (open reading frames) of all available South American and North American isolates, and compared the South American strains of EEEV and VEEV (Venezuelan equine encephalitis virus) phylogenetically.

To explore the evolutionary history of the EEEV complex, a Bayesian coalescent analysis was undertaken. Depending on the data set used, median estimates of when the North and South American lineages last shared a common ancestor were approximately 1600 and 2300 years ago. The genomic distinctiveness between North American and South American lineages was paralleled in their phylogenetic and ecological patterns. The North American strains of EEEV were highly conserved, monophyletic and geographically static, in stark contrast with the highly divergent, polyphyletic, co-circulating and geographically associated relationships among South American strains. It was surmised that the avian hosts on which the North American strains feed are highly mobile and can disperse the virus, whereas the forest-dwelling birds and marsupials that are the hosts of South American strains are not highly motile.

Noting the definition of a virus species by the ICTV (Section 43.1.2) to be a 'polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche', Arrigo *et al.* (2010) suggested that the North American and South American lineages of EEEV be considered independent species within an EEEV complex.

45.1.2 Natural hosts of EEEV

(a) Nearctic Region

Vertebrate hosts:

Enzootic and epornithic cycles: birds, mainly Passeriformes and Ciconiiformes

Dead-end hosts: exotic birds, horses, deer, humans, pigs.

Mosquito hosts and established or putative vectors:

Enzootic cycle: *Culiseta melanura*, *Culiseta morsitans*, *Culex erraticus*.

Epornithic cycle: *Coquillettidia perturbans*, *Ochlerotatus canadensis*, *Ochlerotatus sollicitans*, *Culex peccator*, *Culex salinarius*, *Aedimorphus vexans*, *Uranotaenia sapphirina*.

(b) Neotropical Region

Vertebrate hosts

Enzootic cycle: birds, including forest birds of many species; Didelphidae (opossums), *Didelphis marsupialis*, *Philander opossum*; Reptiles (lizards)

Dead-end hosts: horses.

Mosquito hosts and putative vectors

Culex (*Melanoconion*): *Culex dunni*, *Culex panocossa*, *Culex pedroi*, *Culex taeniopus*

Aedimorphus: *Aedimorphus vexans*.

Mosquito systematics: *Culex* (*Melanoconion*): Sirivanakarn (1983); Pecor *et al.* (1992); Sallum and

Forattini (1996); Navarro and Weaver (2004); Harbach, 2011b,c). *Aedimorphus* (Reinert *et al.*, 2009). See Section 45.1.7 for identification of putative vertebrate hosts and mosquito vectors in the Neotropical Region.

(c) Experimental transmission

In the laboratory, *Culiseta melanura* showed vectorial competence for EEEV. Females that fed on chicks with viraemias of $5 \times 10^{4.5}$ to $5 \times 10^{7.5}$ LD_{50} ml^{-1} became infected and later transmitted the virus to healthy chicks (Howard and Wallis, 1974). When infective *Cs. melanura* fed individually on European starlings (*Sturnus vulgaris*) or American robins (*Turdus migratorius*), the birds' viraemias were most intense on the following day, at $10^{7.3}$ and $10^{5.7}$ PFU ml^{-1}, respectively. In starlings, the viraemia remained of sufficient titre to infect mosquitoes for 3 days, compared with just 1 day in robins, thus showing greater 'amplifying competence' (Komar *et al.*, 1999).

Inoculation of white ibis (*Eudocimus albus*) and snowy egrets (*Egretta thula*) with low doses of EEEV led to viraemias of about 48 h duration, with peak titres of $10^{3.5}$–$10^{5.8}$ and $10^{3.4}$–$10^{4.8}$ mouse $ICLD_{50}$ (intracerebral LD_{50}), respectively. The birds did not appear unwell during the viraemias, and survived well. Neutralizing antibodies appeared after a short time. Among smaller birds infected in the laboratory (cardinal, *Cardinalis cardinalis*; red-winged blackbird *Agelaius phoeniceus*; and sparrow, *Passer domesticus*), all showed very high viraemia, but fatality rates were high (Kissling *et al.*, 1954b).

The susceptibility of horses to infection with EEEV was measured by subcutaneous inoculation of horses aged 1–12 years. Viraemias did not develop in horses receiving a dose of $10^{2.2}$ mouse $ICLD_{50}$ or less, but viraemias of up to $10^{3.8}$ $ICLD_{50}$, lasting 24–66 h, were found in horses receiving $10^{2.5\text{-}4.5}$ $ICLD_{50}$. In four attempts to infect horses by exposing them to batches of 12–58 infected *St. aegypti*, only one horse exposed to 48 mosquitoes became infected. Attempts to transmit EEEV from that horse to two uninfected horses, using a total of 436 mosquitoes, were unsuccessful (Kissling *et al.*, 1954a). Similar results were obtained by Sudia *et al.* (1956). In one exceptional case, a horse that had been inoculated with $10^{4.4}$ $ICLD_{50}$ EEEV had viraemias as high as $10^{5.5}$ $ICLD_{50}$ ml^{-1} during periods of fever. When batches of *Oc. sollicitans* fed on the horse during periods of raised body temperature, some 14–41% of females became infected. After 2 weeks of extrinsic incubation, infective mosquitoes were fed on a healthy horse, which developed a fatal infection. That animal developed a lower-titred viraemia, more characteristic of horses. Mosquitoes were fed on the second horse when its body temperature was raised, a few hours after the brief peak viraemia of $10^{3.2}$, but none became infected. The laboratory findings are consistent with the generally held view that horses are dead-end hosts of EEEV. However, horses can be attacked by hundreds or even thousands of mosquitoes in a day (Gould *et al.*, 1964), and high natural inoculation rates might result in high-titred viraemias. After females of *Oc. sollicitans* had become infected with EEEV by feeding on a viraemic horse, and had survived through the period of extrinsic incubation, up to 500 times more virus was recovered from some individuals than they had ingested (Sudia *et al.*, 1956). The immunity induced by horse vaccines for EEE is short-lived (Weaver, 2001, review), and equine cases continue to appear.

45.1.3 Enzootic and epornithic transmission cycles in the Nearctic Region

Eastern equine encephalitis occurs along the Atlantic coast of the USA, from Massachusetts to Florida, and along the Gulf coast to eastern Texas (cf. Figure 45.1). Inland, foci of EEE occur in two Canadian provinces and seven American states. Within those broadly defined areas, the occurrence of EEE is patchy, the patchiness resulting largely from the habitat requirements and behaviour of the main enzootic vector. Within most of its coastal and inland foci, EEEV is maintained in enzootic cycles involving birds and *Culiseta melanura*. The habitats in which enzootic transmission cycles occur are forested, freshwater swamps, i.e. areas of wet, spongy ground that are saturated or intermittently inundated by standing water, that are

without surface peat, and that typically are dominated by woody plants. Each year, enzootic transmission occurs on the coast of New Jersey and Maryland, where swamps wooded with white cypress (*Chamaecyparis thyoides*) drain into salt marsh. At the inland foci of EEEV, the transmission cycles are also based in swamps. All of these habitats have a large and species-rich avian fauna.

Culiseta melanura satisfies the criteria needed to establish that a mosquito species is a natural vector (cf. Section 44.2.3). Certain life-cycle and behavioural characteristics of *Cs. melanura* determine its effectiveness as a host and vector of EEEV, so its biology is described below at some length. Findings on enzootic cycles of EEEV at coastal foci in Maryland and New Jersey, and at inland foci in central New York State and central Alabama, are described. At some locations in the southern states, *Cs. melanura* is insignificant as host and vector of EEEV, and other species have that role (Subsection 45.1.3.e below).

(a) Culiseta melanura

Broadly, *Culiseta melanura* is found in south-eastern provinces of Canada and eastern states of the USA. It is less common in southern states bordering the Gulf of Mexico, where its presence during the past 100 years has been affected by deforestation for farming and, in places, by a return to forested swamp (Cupp *et al.*, 2004a). Darsie and Ward (2005) show a distribution that extends west to Arkansas and Oklahoma.

Culiseta melanura larvae develop in freshwater wooded swamps or sphagnum bogs with a water table that remains at or near the soil surface through most of the year, and where uprooting of trees leads to formation of water-filled depressions or cavities. The swamps are often, but not invariably, well shaded. The larvae may be found in water-filled depressions, but mostly they inhabit subterranean cavities, e.g. 'swamp crypts' – cool, dark pockets of water that form beneath buttressed swamp trees, extending 1–2 m below ground, or 'earth cavities' which form under trees that have been partly windthrown or at the sites of rotted pine stumps. Larvae also occur in rot holes in trees, but only where the rot hole is at ground level and partly filled with water percolating in from outside. The prime characteristics of these sites are darkness, contact with soil and low water temperature (Silverly and Schoof, 1962; Joseph and Bickley, 1969). Trees having root systems that can produce suitable larval habitats for *Cs. melanura* include white cypress, red maple (*Acer rubrum*), American hornbeam (*Carpinus caroliniana*) and loblolly pine (*Pinus taeda*) (Flemings *et al.*, 1960; Joseph and Bickley, 1969; Weaver, 2001).

At the natural temperatures of the cool-temperate region, the aquatic stages of *Cx. melanura* develop slowly. In Maryland, one generation arose from eggs laid during late summer or autumn, and overwintered in the larval stage. By spring most overwintering larvae were in the fourth instar, and water temperatures governed the dates on which the adults would emerge in late spring or early summer. Eggs laid by those mosquitoes gave rise to the second generation, the adults of which emerged some 3 months later. Their progeny overwintered as larvae (Muul *et al.*, 1975; Mahmood and Crans, 1998). *Culiseta melanura* was found to be bivoltine at a site at 38° N in Maryland (Joseph and Bickley, 1969), so it is improbable that at 43° N in New York State it could be trivoltine, as Morris *et al.* (1976) suggested.

A number of investigators have reported that *Cs. melanura* feeds very largely on birds. A detailed study at a swamp near Vero Beach, Florida, by Edman *et al.* (1972) showed the host sources of captured, engorged females to be, by class: birds, 98.9%; mammals, 0.7%; reptiles, 0.5% (n = 1420). Identification of the avian blood meals to orders of bird hosts showed that, at a time when passeriforms constituted 85.7% of the avian fauna of the swamp, they had contributed 79.2% of avian blood meals. Therefore, the forage ratio (or host preference) for passeriforms was 0.92. The forage ratio for the much smaller ciconiiform population was 3.04. Forage ratios significantly greater than 1 indicate selective preference for a host species or host type (Volume 2, Sections 39.2.2, 39.3.3.b).

At a location in Massachusetts, host-finding activity by *Cs. melanura* started shortly after sunset and continued at a relatively constant level throughout the night. Catches in light traps operated at heights of 1.5, 4.5 and 7.6 m showed that flight activity was the same at all elevations. Avian blood was identified in 98.7% of blood meals at the edge of a swamp, and in 98.8% outside the swamp. Early in the season the great majority of blood meals were from passerines, but from mid-summer onwards the proportions from non-passerine birds and mammals increased. At one site at the swamp edge, 11.5% of the total meals were mixed, often consisting of blood from passerine birds and rabbits (Nasci and Edman, 1981a,b).

Two important characteristics of *Cs. melanura* as host and vector of EEEV are the restricted nature of its larval habitat, i.e. forested swamps, and the strong preference of the adult females for birds.

(b) Pocomoke Swamp, Maryland

Over a number of years, investigations into arbovirus transmission were undertaken at Pocomoke Cypress Swamp, eastern Maryland (Figure 45.2). The swamp consisted largely of secondary growth on a ground base of closely intertwined roots, leaves and other organic material in varying degrees of decomposition, so-called 'closed root-mat swamp'. It included areas of lower-lying, continuously wet substratum (open swamp), and was bordered by slightly higher ground (so-called uplands). Overall, the study site included areas of closed-root mat swamp, open swamp, marsh,

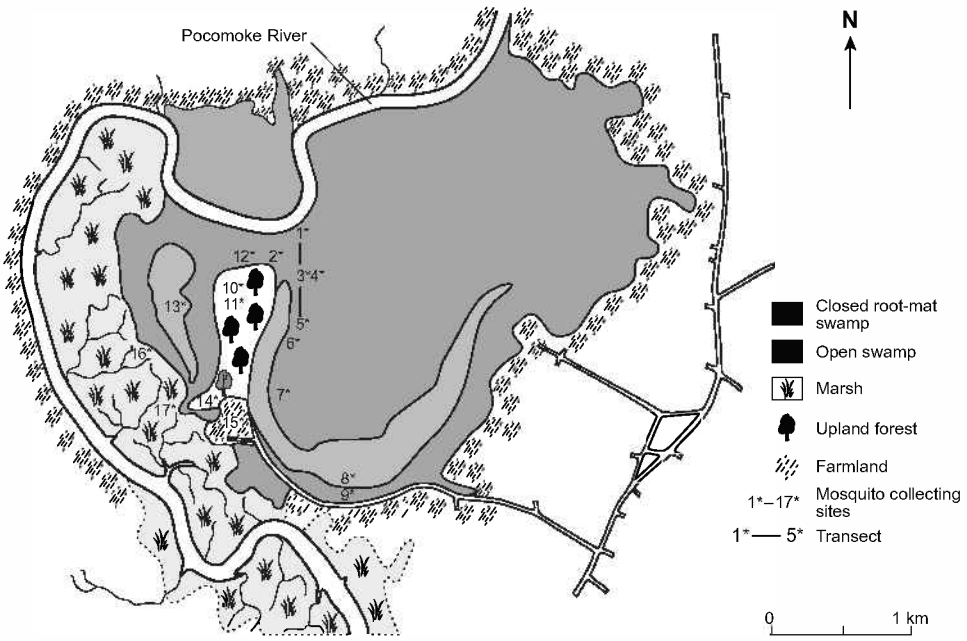

Figure 45.2 Map of the Pocomoke Swamp, Maryland, located at *c.* 38° 03' N, 75° 35' W. (Redrawn from Saugstad *et al.*, 1972.) The swamp is situated in a bend of the Pocomoke River, on the Delmarva peninsula of eastern Maryland. The Pocomoke Swamp, about 9.75 km^2 in area, forms part of a swamp system that extends along the Pocomoke River. It is bordered on the south, east and north by farmland and the Pocomoke River, and on the west and south-west by a marsh which is inundated twice daily as the river level fluctuates tidally. The salinity of the surface water of the swamp is low (18–21 ppm). Previously, much cypress had been removed from the Pocomoke Swamp, and at the time of the investigations it was a secondary growth maple swamp with a marked understorey. A forested area of mixed hardwood and conifer on slightly higher ground projects into the swamp. The course of a transect running from the river edge into the swamp is marked.

'upland' forest and farmland. It had rich mosquito and bird faunas and supported a number of mammal and reptile species. Several arboviruses were present in the swamp, including three alphaviruses: Keystone virus, which was transmitted by *Ochlerotatus atlanticus* (Section 41.4.2.a); Eastern equine encephalitis virus; and Highlands J virus (HJV), a pathogen of domesticated birds. During these investigations, Highlands J virus was misidentified and reported as Western equine encephalitis virus (Karabatsos *et al.*, 1988).

During 1968 and 1969, the following sampling and analytical tests were undertaken. (i) Dipping for and trapping mosquito larvae, to establish distribution and densities. Larvae were also sought in holes dug into the root mat, especially into crypts. (ii) Trapping with light traps and bird-baited traps. (iii) Vacuum sweeping, to establish the distribution and densities of adult mosquitoes. (iv) Screening of captured mosquitoes for EEEV or HJV. (v) Screening of sentinel birds for antibodies against EEEV and HJV (a sentinel animal is one deliberately placed in a particular environment to detect the presence of an infectious agent, such as a virus). (vi) Screening of wild birds caught in mist nets for EEEV and HJV, and for seropositivity (Williams *et al.*, 1971; Saugstad *et al.*, 1972; Muul *et al.*, 1975).

Thirty-four species of mosquito inhabiting Pocomoke Swamp and adjacent areas were identified. Concerning mosquito larvae, collections made in swamp, marsh and forest at 1- or 2-week intervals throughout 1969 yielded 9718 larvae of the 34 species. The three most abundant species were *Oc. canadensis* (36.9%), *Cs. melanura* (16.1%) and *Cx. salinarius* (10.7%) (Saugstad *et al.*, 1972). The collection rates per unit area for *Cs. melanura* larvae were highest in the closed root-mat region of the swamp. Collecting along a transect through that region revealed that larval densities were almost zero within 140 m of the river but became progressively higher further into the swamp. Larvae of *Cs. melanura* were also found in rot holes in the upland forest, but the density per unit area was low.

Light trapping and other methods yielded a total of 346,691 adult mosquitoes. As with the larval collections, the three most abundant species were *Oc. canadensis* (42.8%), *Cs. melanura* (28.6%) and *Cx. salinarius* (13.0%) (Saugstad *et al.*, 1972). Precipitin tests of the gut contents of engorged mosquitoes captured in the swamp from May to November 1969 revealed the host-feeding patterns of different species (Table 45.1). As at other locations in North America, *Cs. melanura* had fed almost entirely on birds. *Ochlerotatus canadensis* and *Cx. salinarius*, which were putative bridge vectors, seldom fed on birds, and showed a preference for mammals or for mammals and reptiles (LeDuc *et al.*, 1972).

Certain investigators asserted that adult *Cs. melanura* were more abundant in the swamp than in the upland forest and cultivated areas, corresponding with the distribution of larvae and availability of birds, and that this distribution was a major factor in the confinement of EEEV to swamp habitats during non-epizootic periods (Williams *et al.*, 1971; Saugstad *et al.*, 1972). However, Morris *et al.* (1980b) challenged these assertions. In an earlier study, Joseph and Bickley (1969) had captured more unfed and engorged females in resting boxes on farms near Pocomoke Swamp than at the swamp. Possibly, female *Cs. melanura* feed on birds both at the swamp and in

Table 45.1 Summary of the host-feeding patterns of three species of mosquito captured at and around Pocomoke Swamp, Maryland, May to November 1969, as shown by analyses of the gut contents of engorged females. (From the data of LeDuc et al., 1972.) Engorged females were captured in light traps and resting boxes, and by net sweeps. *Culex salinarius* was most often captured near farm animals outside the swamp. Of blood meals taken on mammals by *Ochlerotatus canadensis*, 69% were on deer. In spring, when turtles left the swamp to lay eggs on higher ground, the proportion of meals of reptile blood in *Oc. canadensis* increased substantially.

Source of blood meal	Culiseta melanura (%)	Ochlerotatus canadensis (%)	Culex salinarius (%)
Birds	98.5	6.1	6.2
Reptiles	0	16.9	0
Mammals	1.5	77.0	93.8
No. of animals	335	669	32

surrounding upland areas, and gravid females eventually return to the swamp to oviposit.

During May to October 1968, 991 birds captured in mist nets in the Pocomoke Swamp were tested for EEEV and HJV, and for antibodies to those viruses. EEEV was isolated from 11 birds, and anti-EEEV antibodies were found in 120 birds (12.1%). HJV was isolated from two birds, and anti-HJV antibodies were found in 71 birds (7.2%) (Williams *et al.*, 1974). During May to October 1969, when 2866 birds of 77 species were similarly tested, EEEV was isolated from five birds, and HJV was isolated from four. The prevalence of antibodies to the two viruses is shown in Table 45.2.

Only five isolates of EEEV were obtained, all between 22 September and 20 October, the time of onset of the autumn migration. EEEV was isolated from each of three swamp sparrows (*Melospiza georgiana*) in southwards migration, suggesting a possible mechanism for the late introduction of EEEV into the swamp. No EEEV isolations were made from incoming spring migrants, even though many were captured and screened. Of the 29 bird species, of which 20 or more individuals were screened for EEEV-specific antibodies, 28 provided specimens that were seropositive. The first peak of positivity appeared during the spring migration, and involved principally winter residents and spring migrants. A second peak in September and October occurred during the peak period of virus transmission. Species classed as permanent residents and summer residents had the highest seropositivity rates (Table 45.2). The transients and winter residents, which inhabited other areas during the summer, yielded proportionately fewer seropositive specimens when captured during their passage through the swamp. The antibody profiles of summer residents examined when leaving the swamp in 1968 and when returning in the spring of 1969 were essentially the same, suggesting an absence of continued virus transmission in southern wintering areas (Dalrymple *et al.*, 1972).

In 1968 and 1969, *Cs. melanura* was the only mosquito from which EEEV or HJV was isolated. In 1968, HJV was first detected on 29 July, and EEEV on 6 August. During the period of mosquito infection (broadly August to October 1968), the mean MIRs (minimum infection rates)/1000 were: HJV, 1.2 (range 0.52–6.6); and EEEV, 2.0 (range 1.2–3.4). Almost all EEEV isolations were from the swamp area, whereas HJV isolations were more widely spread, including upland forest. In 1968, neutralizing antibodies to HJV and EEEV appeared in sentinel bobwhite quail (*Cotinus virginianus*) on the same dates that the viruses were first isolated from mosquitoes: 29 July for HJV, 6 August for EEEV (although samples were not taken daily). Subsequently, the seropositivity rates rose rapidly, and by 20 August, within the swamp, 80% of sentinel birds were seropositive for antibodies to EEEV (Williams *et al.*, 1972, 1974). In 1969, HJV was again first detected in *Cs. melanura* in July, but

Table 45.2 Seropositivity rates for antibodies against Eastern equine encephalitis virus and Highland J virus (HJV) in wild birds in Pocomoke Swamp, Maryland, during 1969, grouped in four classes according to the birds' residential status. (From Dalrymple *et al.*, 1972, who cited HJV as WEEV.)

Class	*Wild birds*		*Proportions positive for*	
	Total (n)	*Antibody positive* (n)	*EEEV* (%)	*HJV* (%)
Transient	632	59	2	8
Winter resident	707	130	7	12
Permanent resident	743	304	32	26
Summer resident	784	355	28	21
Totals	2866	848		

The characteristics of the classes of birds, as described for New Jersey populations by Boyle (1994) and cited by Mahmood and Crans (1998), are as follows. **Permanent residents.** Remain year round in the one region. **Summer residents.** In May fly northwards from wintering sites in the tropics to join permanent residents. Both groups establish nesting sites in wooded habitats during May to raise their young. The fledglings of both permanent and summer resident species appear during June in New Jersey and remain on site into the autumn. In September, the summer resident species fly south to their preferred winter range. **Winter residents.** These species join the permanent residents during October and remain until spring. **Transients.** Species that nest further north than New Jersey and that start to migrate during August, when they move through the area continuously, replacing local populations.

serological evidence from sentinel quail indicated that transmission was occurring from mid-June onwards. The dates of detection of EEEV in *Cs. melanura* were 19–25 August to 9–14 September, during which period the mean MIR was 0.43. Infection of sentinel quail occurred from the beginning of August (Dalrymple *et al.*, 1972; Saugstad *et al.*, 1972).

These various findings pointed to the existence of an enzootic cycle for EEEV, involving *Cs. melanura* and birds, in and around Pocomoke Swamp (Figure 45.3).

(c) Swamp near Dennisville, New Jersey

Studies of the ecology and transmission of EEEV were undertaken at a site about 3 km south-west of Dennisville, New Jersey (*c.* 39° 10' N, 74° 50' W). The site was formed by a 1.3 km long peninsula that extended from an upland forest to a salt marsh, and was bordered on either side by a large white cypress swamp. The peninsula provided five distinct habitats: (i) upland forest of white oak (*Quercus alba*), black oak (*Quercus velutina*), pitch pine (*Pinus rigida*) and other species; (ii) lowland deciduous forest of red maple (*Acer rubrum*) and other species; (iii) a mature white pine (*Pinus strobus*) plantation; (iv) a dying red pine (*Pinus resinosa*) plantation; and (v) a 1 ha field. Mosquitoes were collected from 1975 to 1984, and from 1979 to 1984 were screened for virus. For periods of about 6 months in 1980 to 1983, birds were captured in mist nets, bled, and screened for EEEV and anti-EEEV antibodies. From 1981 to 1983, captured birds were ringed to permit identification of recaptures (Crans *et al.*, 1994).

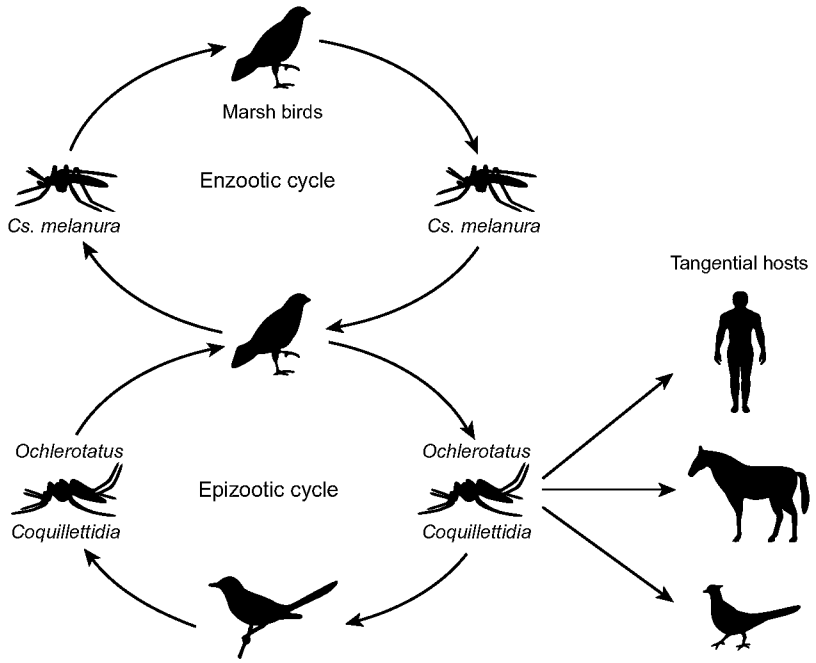

Figure 45.3 Transmission cycles of Eastern equine encephalitis virus in north-eastern North America. (After Johnson, 1998, with modification.) The enzootic cycle, which occurs in freshwater marshes, is between birds and the mosquito *Culiseta melanura*. Away from marshes, the virus can enter an epizootic cycle, when certain species of *Ochlerotatus*, *Coquillettidia* or *Culex* transmit it to other wild birds. The same epizootic vectors may function as bridge vectors and transmit the virus to tangential (dead-end) hosts. Some authors use the term 'epornithic' for transmission cycles in which the vertebrate hosts are birds.

During the 6-year period 1979 to 1984, a total of 48,510 *Culiseta melanura* were captured and screened for EEEV. In 1983, only one isolation of EEEV was made, but in the other years the mean monthly MIRs during the periods of active transmission ranged from 1.2 to 5.6. In 3 of the 6 years, the first isolations were made in July; in the other 3 years they were made during August. The MIRs peaked in September or October (Figure 45.4).

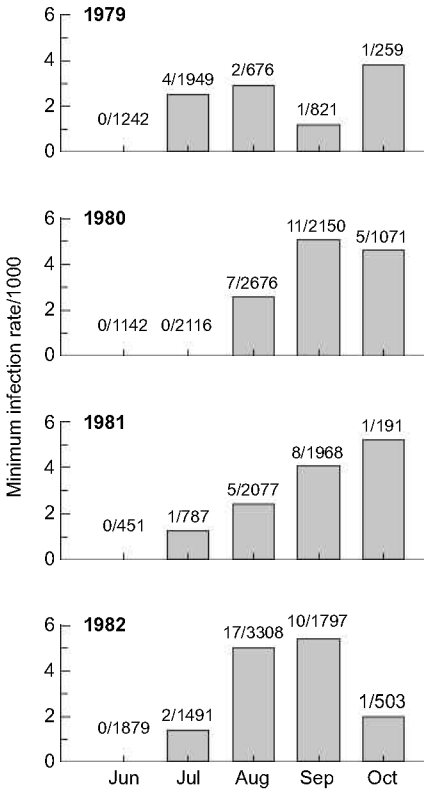

Figure 45.4 Histograms representing the minimum infection rates of Eastern equine encephalitis virus in *Culiseta melanura* at a swamp near Dennisville, New Jersey, month by month during the years 1979 to 1982. (After Crans *et al.*, 1994.) The numbers above each bar indicate number of positive pools/total number of mosquitoes tested. Not shown are the results from 1983, when only a single isolate was obtained (in August), and 1984, when isolates were obtained in August, September and October.

Of 1848 birds captured in 1980–1982 and submitted to virological tests, 19 (1.03%) were viraemic with EEEV at the time of capture. In 1980, the dates of isolation of EEEV from mosquitoes and birds were similar. In 1981, one viraemic bird was captured 7 days before the first infected mosquito, but the mosquitoes were sampled only at weekly intervals. In 1982, five viraemic birds were captured in May or June, some 37–51 days before the first isolations from mosquitoes. Of these birds, two were classed as summer residents and three as partial migrants. During 1980–1983, 494 birds of 69 species were screened for anti-EEEV antibodies. Some 27% of the birds were seropositive, representing 68% of species. Usually, seropositivity levels were relatively high in spring, but decreased in May or June before rising again as the season progressed, peaking in late summer. During May, seropositive samples came entirely from birds that had hatched in earlier years. Birds trapped during the year in which they had hatched were first captured in June. The proportion of such juveniles rose steadily from about 20% in early June to 60% in late September. Of 816 birds screened in the year of hatching, 27.7% had antibody to EEEV at the time of capture. The date of capture for 6.6% of these birds preceded the date of earliest virus detection in mosquitoes. When grouped according to residency status, the birds showed the following seropositivity rates: permanent residents, 37.1%; summer residents, 33.2%; partial migrants, 25.4%; migrants, 8.6%; winter residents, 5.8% (Figure 45.5). Among the birds identified as recaptures, 29 had seroconverted since their earlier capture; ten of these showed evidence of seroconversion 8–56 days before first detection of virus in *Cs. melanura*. Resident status was the only characteristic that showed strong association with the EEEV transmission cycle. Crans *et al.* (1994) postulated that a cryptic transmission cycle develops in early spring, weeks before detection of EEEV in *Cs. melanura*, and surmised that changes in the physiological state of local birds in early spring may stimulate reactivation of latent virus. However, they conceded that much additional information was needed to validate their hypothesis.

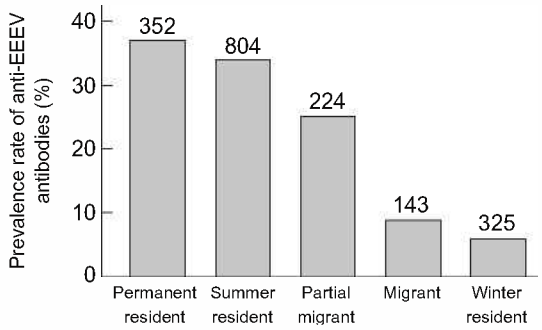

Figure 45.5 The prevalence rate for anti-EEEV antibodies in birds captured at a swamp near Dennisville, New Jersey, grouped according to their residency status. (After Crans *et al.*, 1994.) Sample size is indicated above each bar. Antibodies were identified by haemagglutination inhibition followed by plaque reduction neutralization tests.

(d) Toad Harbor Swamp, Central New York State

Eastern equine encephalitis has occurred in three regions of New York State: within Long Island; within the lower Hudson Valley; and in a group of four counties in central (upstate) New York (Howard *et al.*, 1994). In this section, interest is focused on the third of those regions, an area of 2600 km^2 within four counties (Oswego, Oneida, Madison and Onondaga) that surround Oneida Lake (Figure 45.6), where numerous infections in horses have been recorded. Three established enzootic foci of EEEV are known, Toad Harbor Swamp and Big Bay Swamp in Oswego County and Cicero Swamp in Onondaga County. All are hardwood swamps. Toad Harbor Swamp is very close to Big Bay Swamp, and they form the so-called Toad Harbor Swamp Complex, a site of ecological investigations. Together, they extend over more than 2000 ha, along the northern shore of Oneida Lake. The uplands that extend away from the two swamps on their other sides include woodland, horse pasture and a village 2 km from the swamp edge. In 1971, investigations into the transmission of EEEV in upstate New York commenced at the Toad Harbor Swamp complex (Morris *et al.*, 1973, 1980a,b). In 1978, an Encephalitis Field Station was built on the edge of that swamp, and virological screening of mosquitoes was conducted routinely from that time. EEEV transmission in central New York State is thought to be independent of transmission along the eastern seaboard. In contrast to

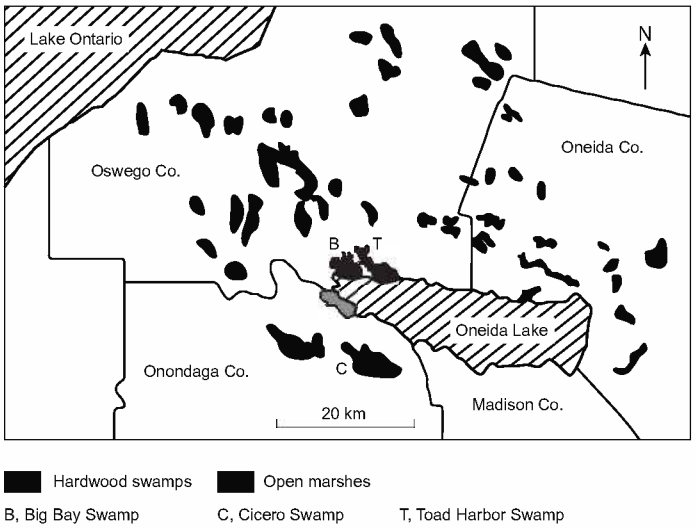

Figure 45.6 Map of a region of central New York State where equine cases of Eastern equine encephalitis occur, showing the locations of the many hardwood swamps. (After Howard *et al.*, 1996.) The two known foci of epornithic transmission of EEEV are the Toad Harbor–Big Bay Swamp complex and Cicero Swamp.

the coastal foci, EEEV is not detected each year (Emord and Morris, 1984; Howard *et al.*, 2004). Highlands J virus is also found at Toad Harbor Swamp, where it can cause enzootics.

Mosquitoes. During 1977 to 1979, sampling with CO_2-baited light traps and resting shelters at the Toad Harbor Swamp complex showed both sexes of *Cs. melanura* to be more abundant at the swamp centre during May and June, and more abundant at the swamp edge thereafter. The density of females was lower at the village, but a significantly greater proportion of females at the village was parous and either blooded or gravid. Parity data indicated that the females remained in the swamp when populations were low or during adverse weather conditions, but that during periods of high population density or of warm, wet weather the older females dispersed at least as far as the village. Precipitin testing of blood-filled females from all sites showed that 99.7% had fed on birds (n = 2009). Of the females from horse pastures, 100% had fed on birds (Morris *et al.*, 1980b).

Parous rates suggested that female *Cs. melanura* had at least a moderate life expectancy. In 1977, among non-blood-fed females captured in light traps, the parous rate was 38% in June and 51% in July. Later, possibly with a new generation of adults, it increased from 3% in the first week of August to 33% in the third (Morris and Srihongse, 1978).

Virological screening of mosquitoes for EEEV started in the first week of June and continued until autumn; the findings for the whole of New York State for the period 1971 to 1992 are shown in Figure 45.7D. The mean number of isolations of EEEV from mosquitoes by month was: June, 3; July, 49; August, 87; September, 52; and October, 9 (Howard *et al.*, 1988, 1994). Virological screening during 1990 of mosquitoes collected at a number of sites in central New York State produced 86 isolations of EEEV; in 1991, 40 isolations were made. For the 2 years combined, the numbers of isolations per species were: *Cs. melanura*, 103; *Cs. morsitans*, 6; *Cq. perturbans*, 10; *Oc. canadensis*, 4; *Am. vexans*, 2; and *Anopheles quadrimaculatus*, 1 (Howard *et al.*, 1996). During 1990 at Toad Harbor Swamp, 33 isolations were made: *Cs. melanura*, 26; *Cs. morsitans*, 4; *Oc. canadensis*, 2; and *Cq. perturbans*, 1. *Culiseta melanura* and *Cs. morsitans* were considered to be enzootic vectors, and *Cq. perturbans* and *Oc. canadensis* to be epornithic/epizootic vectors (Howard *et al.*, 1988, 1994, 2004).

Birds. The findings from screening wild birds and sentinel pheasants for EEEV in New York State as a whole for the period 1971 to 1992 are shown in Figure 45.7C. Epornithics of EEE occurred at the Toad Harbor Swamp complex in 1971, 1976, 1977, 1988 and 1990, permitting investigations into infections in wild birds which are reported in some detail here. During the 1971 epornithic, seropositivity for EEEV was detected in six of 20 bird species collected in Oswego County (n = 93); virological tests on 19 of the species detected EEEV in four. (Sera positive by the HI (haemagglutination inhibition) test were checked by neutralization tests.) Seropositivity rates were highest among birds captured near the Toad Harbor Swamp complex, notably in Baltimore orioles (*Icterus galbula*) and white-throated sparrows (*Zonotrichia albicollis*). Patterns of capture of the Baltimore orioles, and the young age of the white-throated sparrows, suggested to Morris *et al.* (1973) that EEEV was brought into the area by southwards-moving birds. A serological study conducted during 1978–1980, an inter-epornithic period, led to a different conclusion. During this study it was established that HI titres of 1:160 could persist for at least 3 years post-infection. Measured weekly, the antibody prevalence rate peaked during the breeding season, and decreased as young susceptible birds fledged and as migrants replaced breeders. Further, antibody prevalence was significantly higher in ringed birds that were recaptured in more than 1 year than in that of the general population, suggesting that the birds that had returned yearly to the site were those most likely to have been on site during the 1976 and 1977 epornithics. Emord and Morris (1984) concluded that local-breeding birds probably are infected on site, and that birds do not reintroduce the virus during northern and southern migrations.

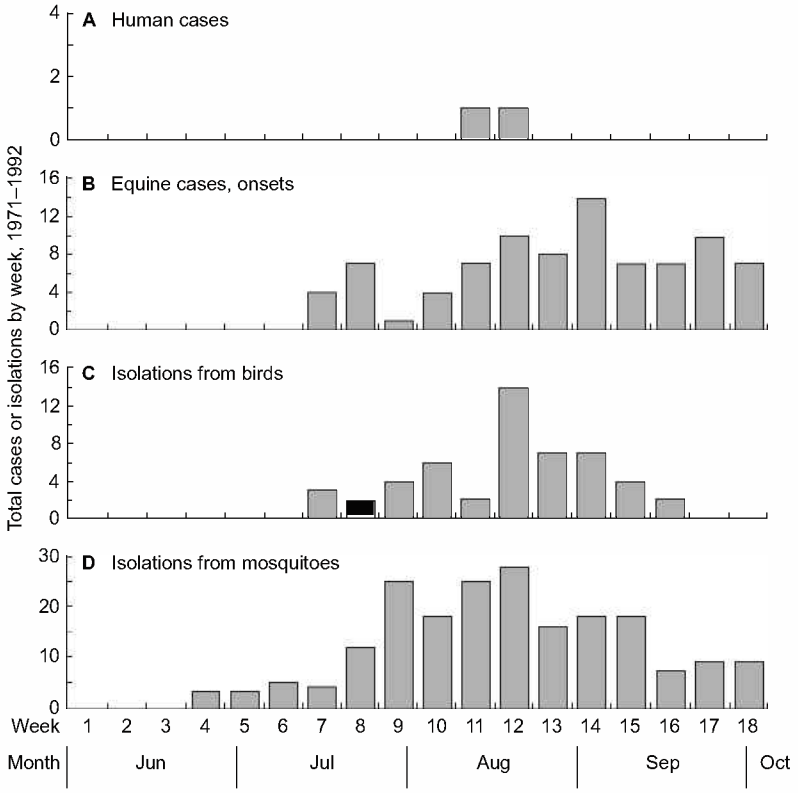

Figure 45.7 The seasonality of Eastern equine encephalitis in humans (A) and horses (B), and of isolations of Eastern equine encephalitis virus from birds (wild birds and sentinel pheasants) (C) and mosquitoes (D), based on records from central New York State for the period 1971 to 1992. Screening of birds and mosquitoes started in the first week of June. (After Howard *et al.*, 1994.)

During the period 1986–1990, birds of 35 species were found positive for anti-EEEV antibodies. The proportions of blood samples that were seropositive were: 10% in 1986; 9.2% in 1987; 7.3% in 1988; 23% in 1989; and 31.6% in 1990. During the summers of 1986 to 1990, mist netting was carried out weekly. Of the 99 species captured (n = 6926), five species constituted 53% of all captures. All ringed birds captured had previously been ringed on site. For screening, 4174 birds were bled, 60.3% of the total. Epornithics occurred in 1988 and 1990, and only in those years was EEEV isolated. In 1988, EEEV was isolated from five birds bled between 22 August and 13 September. In 1990, EEEV was isolated from 25 birds bled between 16 July and 3 September. The 30 isolations were from birds of 12 species; 26 of the isolations were from birds that had hatched during the year of capture. No evidence was found of viral recrudescence in returning birds that had hatched locally in earlier years, or of virus being introduced by birds on southward migration. Howard *et al.* (2004) concluded that the question of whether EEEV is consistently present in some cryptic form, annually introduced as a chronic infection in birds, or sporadically introduced with migrating birds remains unanswered for the foci in central New York State.

Horses. In central New York State during the 21-year period 1971 to 1992, there were 92 equine cases of EEE and two human cases (both fatal) (Figure 45.7A,B). The outbreaks in horses were spasmodic: peaks of activity occurred in 1974 (11 cases), 1976 (40 cases) and 1983 (nine cases); inter-

epizootic periods with no cases occurred in 1977–1981 and 1983–1989. During the 21-year period, the mean distribution of the 92 equine cases by month was: July, 13; August, 30; September, 41; and October, 8 (Howard *et al.*, 1988, 1994). A possible association between number of equine cases and weather is detailed in Section 45.1.4.

(e) South-eastern states

Cases of equine EEE still occur in considerable numbers in southern states of the USA. Infections with EEEV have been reported from birds, and at certain foci additional mosquito species have been identified as hosts and putative vectors of EEEV. We examine reports from both coastal and inland, or unspecified, locations.

In **Louisiana**, serological evidence suggested that ciconiiform birds might be amplifying hosts. At an unspecified location, high proportions of the yellow-crowned night heron (*Nictanassa violacea*) were seropositive: in 1952–1953 (April through to June), 80% (n = 30); in March 1956, within 1 week of their return from South and Central America, 90% (n = 49). Unlike that of many smaller birds, the (?) average age of the breeding population of this species was at least 3 years, permitting exposure during several seasons. Yellow-crowned night herons nested only on the edges of permanent 'deep holes of water', sites that provided aquatic habitats for *Culiseta melanura*. Their nestlings stood quietly and allowed scores of mosquitoes to feed on them, while nearby the nestlings of little blue heron, green heron and Louisiana heron showed defensive behaviour. When measured at the same times, seropositivity rates in the yellow-crowned night heron were much higher than those in the Louisiana heron and little blue heron (Stamm, 1958).

At mostly coastal locations in Louisiana, a number of ciconiiform species were seropositive for EEEV-specific neutralizing antibodies, namely: white ibis (*Eudocimus albus*); snowy egret (*Egretta thula*); little blue heron (*Egretta caerulea*); eastern green heron (*Butorides virescens*); and black-crowned night heron (*Nycticorax nycticorax*). Seropositivity was recorded also in the red-winged blackbird (*Agelaius phoeniceus*) and EEEV was isolated from a purple grackle (*Quiscalus quiscula*), both passeriforms. In the laboratory, white ibis, snowy egret, purple grackle, red-winged blackbird, cardinal, sparrow (*Passer domesticus*) and cedar waxwing (*Bombycilla cedrorum*) were all susceptible to infection by inoculation of low doses of EEEV. With the exception of the ibis and egrets, the viraemias attained upon infection were sufficiently high to infect mosquitoes. Infections in the ibis, egrets and purple grackle produced no visible symptoms, but in the other species infections caused by inoculation of captive birds were almost always rapidly fatal. Kissling *et al.* (1954b) concluded that, among the birds examined, the purple grackle was an important amplifying host.

In **Alabama**, between 1959 and 1963, an investigation was undertaken in a 20 ha swamp at the edge of the Tensaw River, not far from the Alabama southern coast. Birds were captured in mist nets on 78 days between November 1959 and December 1960, when 3020 birds were bled and tested for virus. Forty-two isolates of EEEV were made from 16 species of birds, all during the months of September to December. A rapid spread of virus was apparent during September and October 1960, when 36 isolates were obtained from 684 birds. Serological tests showed little or no EEEV transmission from January through to July, but seropositivity was found from September to December, the highest proportions of positives being found in permanent residents, followed in order by winter residents, summer residents and transient species. A substantial proportion of the winter residents were seropositive by December, suggesting that they had arrived before the cessation of virus transmission (Stamm, 1968).

At the same site, eleven isolations of EEEV were obtained from *Cs. melanura*, all during the months of August to November. For the total catch (n = 2158), the MIR/1000 was 5.1; and the three highest monthly MIRs were 10.2, 13.0 and 13.3. Single isolations were obtained from five other species – *Culex nigripalpus*, *Cx. salinarius*, *Am. vexans*, *Cq. perturbans* and *Anopheles crucians*. On this

evidence, *Cs. melanura* was taken to be the enzootic vector. *Culiseta melanura* overwintered in the larval stage (Sudia *et al.*, 1968).

In a part of Tuskegee National Forest, central Alabama, where beaver ponds provided standing water for much of the year, over 20 mosquito species were captured and screened during the years 2001–2003. EEEV was isolated from adult females of six species (Cupp *et al.*, 2003, 2004b; Hassan *et al.*, 2003). Of particular interest was the finding of new hosts and putative vectors, including two species that feed predominantly on reptiles or amphibians. The following conclusions are drawn from the published data. *Culiseta melanura* was uncommon but may have been a subsidiary enzootic vector. Its MIRs were remarkably high (Table 45.3). *Culex* (*Melanoconion*) *erraticus* may have been the main enzootic or epornithic vector, and is a putative bridge vector. It was by far the commonest species captured, and attacked birds and mammals. Its low-to-modest MIRs were based on a huge catch. Blood meals taken by *Culex* (*Melanoconion*) *peccator* were predominantly from reptiles (93%), the remainder from mammals and amphibians. Its remarkably high MIRs were based on moderate numbers. *Uranotaenia sapphirina* had significant MIRs. Only two blood meals taken by *Ur. sapphirina* were identified, both on amphibians. Some species of *Uranotaenia* feed predominantly on amphibians, others feed on amphibians to a substantial extent but not exclusively (Volume 1, Section 39.3.1.g). *Aedimorphus vexans* and *Cq. perturbans* were putative bridge vectors.

Finally, we note two other foci of EEEV. In a 6-year study undertaken in an area of wetland near Iuka, north-eastern **Mississippi**, EEEV was isolated from mosquitoes of six genera. Among the mosquitoes captured (n = 102,479), relative frequencies and infection rates were as follows: *Cx. erraticus*, 51.9% (MIR = 0.48); *Cq. perturbans*, 16.6% (MIR = 0); *An. crucians*, 16.5% (MIR, 0.59); *An. quadrimaculatus*, 9.0% (MIR = 0.31), and *Cs. melanura* <1% (MIR = 0) (Cupp *et al.*, 2004b). In coastal **South Carolina**, EEEV was isolated from single pools of *Cs. melanura* (MIR = 3.8) and *Ochlerotatus taeniorhynchus* (MIR = 0.6) (Ortiz *et al.*, 2003). Mosquitoes that attack horses are of interest as possible bridge vectors. In south-east Louisiana, species that were captured in a horse-baited stable trap, and that showed high engorgement rates, included *Am. vexans*, *Cq. perturbans* and *Culex* (*Melanoconion*) spp. (Samui *et al.*, 2003).

Table 45.3 Characteristics of the females of six mosquito species captured in Tuskegee National Forest, central Alabama, and susceptible to infection with Eastern equine encephalitis virus. (From the data of Cupp *et al.*, 2003, 2004b; and Hassan *et al.*, 2003.)

Species	*Catch rate* *	*Period when infected with EEEV*	*Blood meals (avian)* †	*MIR/1000*			*MIR: total*
	(%)		(%)	*2001*	*2002*	*2003*	(n)
Culex erraticus	54.4	Mid-June to mid-September	48	3.2	0.8	0.36	35,152
Aedimorphus vexans	7.1	June	25	2.2	1.1	–	1,629 ‡
Uranotaenia sapphirina	4.8	June to September	–	5.6	2.6	0.44	3,634
Culex peccator	1.7	–	–	21.5	16.9	0	803
Coquillettidia perturbans	1.4	August	33	9.9	4.5	–	320 ‡
Culiseta melanura	1.2	Late May to mid-September	–	20.2	40.0	–	273 ‡

*, Percentage of total catch in CO_2-baited light traps and vacuum traps in 2001 and 2002 (n = 23,046).
†, Among females in vacuum collections from resting boxes and natural resting sites in 2001 and 2002.
–, Not measured or not recorded.
‡, 2001 only.
MIR, minimum infection rate.

Stamm (1958) identified two patterns of transmission in wild birds in southern and northern states. (i) In some years, EEEV existed in a sylvatic transmission cycle, and the virus progressed through wild bird populations at a rate typical of enzootics. EEEV was isolated from <1% of birds screened, of which 13–22% were seropositive. There were few or no equine or human cases of EEE. (ii) In certain other years, the virus spread through wild bird populations at explosive speed, and additional species were affected. On certain days, EEEV was isolated from as many as 11% of the birds screened, of which 45–54% were seropositive. The numbers of equine and human cases increased. The author surmised that maintenance of EEEV in an enzootic, sylvatic cycle requires the presence of susceptible bird populations of sufficient population density, and a vector population of 'optimum density'. This balance would be upset by a great increase in numbers of either birds or vectors. Entrance into this transmission cycle of other mosquito species of high vectorial competence would lead to an epornithic.

45.1.4 Transmission to dead-end hosts in the Nearctic Region

In North America, outbreaks of Eastern equine encephalitis occur sporadically among dead-end hosts of the virus. Outbreaks have been most apparent from the fatal cases in equines, deer, humans, pigs and farmed birds of different species. As one example, in Massachusetts during the 55-year period 1938–1992, there were totals of 630 equine cases and 72 human cases with 42 deaths (Edman *et al.*, 1993). Pathological examination of cervids showing neurological symptoms revealed infections of EEEV in white-tailed deer (*Odocoileus virginianus*). A single case was found in Georgia in 2001 (Tate *et al.*, 2005), and eight cases in Michigan (Schmitt *et al.*, 2007). At the Toad Harbor Swamp complex in New York State, EEEV was isolated from blood-fed *Cs. melanura* and *Cs. morsitans*. Other tests on those species revealed a proportion of mixed blood meals, composed of both avian and mammalian blood, including blood from white-tailed deer (Molaei *et al.*, 2006).

A high proportion of equine cases occurred within short distances of foci of enzootic EEEV transmission in freshwater swamps. In 1947, in southern Louisiana and adjacent parts of Texas, an estimated 14,334 horses and mules were infected, of which 11,727 died (Scott and Weaver, 1989). In upstate New York during the period 1971 to 1983, 96% of equine cases occurred within 8 km, and 66% within 4 km of swamps, most of which were inhabited by *Cs. melanura*. The two human cases occurred within 3 km of the two largest *Cs. melanura* sites (Howard *et al.*, 1988). In New York State in 1990, when seven cases of equine EEE occurred around Oneida Lake, five of them were in uplands within a few kilometres of freshwater swamps inhabited by *Cs. melanura*, and two occurred some 5 and 10 km away from such swamps. In that year, EEEV was first isolated from wild birds in the week 15–21 July, from *Cs. melanura* in the week 22–28 July, and from other mosquitoes in the week 5–11 August. The first notification of equine disease was in the week 12–18 August (Howard *et al.*, 1996).

It became important to discover the links between epornithic transmission cycles in the swamps and transmission to dead-end hosts some distance away. It was already known that populations of *Cs. melanura* could be highly mobile, the females flying out of swamps, taking blood meals and returning to oviposit (Joseph and Bickley, 1969; Nasci and Edman, 1984). To measure the potential flight range of *Cs. melanura* at Toad Harbour Swamp, at weekly or shorter intervals between mid-June and mid-September 1986 and 1987, wild-caught male and female *Cs. melanura* were marked and released from three sites – one near the swamp edge and two (supposedly) 0.6 and 1.6 km inland from the swamp edge. Marked mosquitoes were recaptured in light traps, bird-baited traps and resting shelters. The maximum distance of recovery from all but one of the releases was 7.8 km, being the same for males and females, although after one release a single female was recaptured at a distance of 9.8 km. Excluding that

result, the mean distances travelled, from different release sites and in different years, were 3.9–4.1 km for females and 1.6–7.1 km for males. From the nine recapture sites, 42.6% of recaptures were made at the site that was most directly downwind from the release points and only 125 m from the swamp (Howard *et al.*, 1989).

With such a flight range, *Cs. melanura* can carry EEEV some distance from swamps; however, it does not attack equines. A postulated epornithic transmission cycle involved non-marsh birds as vertebrate hosts and a number of mosquito species as vectors (Figure 45.3). Such cycles would be initiated with the infection of birds by *Cs. melanura* at sites distant from swamps, but whether *Cs. melanura* contributes to the maintenance of those cycles is not known. Scott and Weaver (1989) listed 21 mosquito species of five genera as having been naturally infected with EEEV in North America, but that provided no indication of a capability for transmission. The following species have been identified as putative epornithic vectors of EEEV; they were capable of transmission in the laboratory, have appropriate host preferences, occur at foci of infection and have been captured infected with EEEV in the field: *Cq. perturbans*, *Oc. canadensis*, *Oc. sollicitans*, *Cx. salinarius* and *Am. vexans* (Crans, 1977; Howard *et al.*, 1988; Vaidyanathan *et al.*, 1997; Moncayo and Edman, 1999; Bosak *et al.*, 2001; Cupp *et al.*, 2003). Probably, certain of these species are bridge vectors, transmitting EEEV to dead-end hosts.

On the basis of the available evidence, Howard *et al.* (1994) suggested that *Cs. melanura* is sufficiently vagile to transfer EEEV from epornithic foci in swamps to upland areas, and that equine and human disease results from the transmission of EEEV by infected *Cs. melanura* to birds that live close to human habitations, which serve as the virus source for local populations of epidemic vectors.

Geographical information system (GIS) technology and remote sensing were applied to an area of Massachusetts where equine and human cases of Eastern equine encephalitis had occurred, with the purpose of identifying landscape features that defined the habitats in which *Cs. melanura* and three of the putative vectors developed. Deciduous wetland was the landscape category that contributed most to the abundance of populations of *Cs. melanura*, *Oc. canadensis* and *Cx. salinarius*. *Coquillettidia perturbans* develops in permanent freshwater marshes, where the larvae puncture the roots of emergent vegetation, but a model could not be devised that would predict its abundance (Moncayo *et al.*, 2000a). Salt-water marshes provided the larval habitat of *Oc. sollicitans*, and in coastal regions some freshwater swamps abut and drain into such marshes.

A number of authors have commented that years when the numbers of human or equine cases of EEE were exceptionally high in a particular region were years of unusually high rainfall or of higher than normal temperature. Hayes and Hess (1964) carried out a retrospective analysis of weather conditions during the years 1934 to 1961, checking for any association with major outbreaks of EEE in horses or humans. In Massachusetts and New Jersey, major outbreaks were associated with a weather pattern characterized by excessive rainfall during the preceding autumn and again during the summer of the outbreak. In Maryland, there had been no major human outbreaks, but there was a reasonable association between the autumn-summer precipitation index and the occurrence of equine outbreaks. In central New York State, some major equine outbreaks appeared to be associated with rainfall; for example, in 1974 when the August rainfall was 15 cm above average, and in 1976, the wettest summer on record (Srihongse *et al.*, 1978; Howard *et al.*, 1988). Analysis of rainfall patterns in coastal Atlantic counties during the period 1983 to 1989 revealed an association between the occurrence of human cases and excess rainfall. The only significant patterns were of excess rainfall occurring either in the year prior to and during the year of the case, or only during the year of the case. Data from local weather stations or from statewide average rainfall figures gave the highest sensitivity (Letson *et al.*, 1993).

45.1.5 Winter and spring populations of EEEV

(a) Evidence of survival in winter

Epizootics and epidemics of EEE occur at sites in North America from later summer to early autumn. To find whether these outbreaks derive from virus that had overwintered locally or that has been introduced from elsewhere, the genetic characteristics of strains of EEEV present at localized sites of occurrence were determined in successive years.

In Connecticut, upstate New York and New Hampshire, genetically characterized strains of EEEV persisted in some localized sites for 2 or more years. A total of 202 isolates obtained from mosquitoes collected at 31 locations in Connecticut from 1996 to 2007 segregated into a number of clades of identical or nearly identical strains. Most clades were recorded during 1 year only, but three clades were detected over 2 consecutive years, and others over 3 years. Viruses collected in upstate New York from 2004 to 2007 formed a genetically homogeneous clade that was perpetuated locally over 4 continuous years. These findings were interpreted as evidence of EEEV overwintering in northern foci (Armstrong *et al.*, 2008).

In a similar study undertaken in central upstate New York and elsewhere, nucleotide sequences comprising the entire E2 (envelope glycoprotein) and a part of the NSP3 (non-structural protein 3) coding region were compared among a total of 42 strains. Of those strains, 29 were isolated during epizootics in central upstate New York – 13 during 1971–1975 and 16 during 2003–2005. A further seven were isolated along the Eastern Seaboard of the USA during 2002–2003, and the remainder were isolated elsewhere. Strong spatio-temporal clustering of EEEV strains was observed; for example, several strains identical in their E2 coding regions were collected in a single focus over the course of several years. The 16 strains isolated during 2003–2005 formed the Onondaga03 clade, while the strains isolated from Oswego County in 1974 and 1975 formed the Oswego74 clade. Collectively, the data indicated that EEEV was perpetuated locally through several winters in upstate New York, with one dominant genotype circulating in each focus. Young *et al.* (2008) concluded that populations of EEEV had overwintered at those sites.

(b) Modes of survival

If EEEV survives through the winter in infections of its main mosquito host and vector *Cs. melanura*, it could only be within vertically infected larvae. Despite the few reports of vertical transmission of EEEV in *Cs. melanura*, the weight of evidence is that the occurrence of vertical transmission in wild populations is rare (Section 44.6.2.c). It is generally considered that EEEV survives through the winter in vertebrate hosts.

Theoretically, EEEV could survive in infected birds of resident species that remain at enzootic foci throughout the winter, or in migrant species that return to those foci in spring or early summer. Findings from Toad Harbour Swamp on the possible annual reintroduction of EEEV by migratory birds were inconclusive (Section 45.1.3.d). Evidence from a site near Dennisville (Section 45.1.3.c) discounted reintroduction by migratory birds, and led to the hypothesis that EEEV persists through the winter in its isolated foci, and that traces of latent virus might be reactivated in the spring. However, studies of WEEV and SLEV (St. Louis encephalitis virus) suggested that the immune response that is induced in birds by infection with an arbovirus during one season persists and prevents reinfection with homologous virus during the following season. Further, it may prevent the recrudescence of infectious virus in chronically infected birds (Section 44.7.1.b). The source of the EEEV that infects susceptible birds or mosquitoes in enzootic foci during the spring or early summer is not known.

(c) Evidence of reintroduction in spring

Two possible means of reintroduction of EEEV to northern habitats in spring have been considered: (i) By infected migrant birds flying north from their

southern habitats. (ii) By vector mosquitoes carried north on winds.

Phylogenetic analyses suggested that epizootics of EEEV had occurred after the introduction of novel genotypes from southern strains. For example, the Onondaga03 strain grouped strongly with the strain FL02b isolated from an ovenbird (*Seiurus aurocapilla*, Passeriformes) in Florida in 2002. This bird was said to reside in Florida and Central America in the winter, and to migrate north as far as Canada in the summer. Young *et al.* (2008) considered it likely that migratory birds are involved in virus trafficking to at least some degree.

As described in detail in Section 44.7.2, analyses of backward trajectories of winds moving from Florida and other southern states northwards over the Eastern Seaboard of the USA as far as New Jersey, Maryland and upper New York State indicated that they could have transported mosquitoes. It was known that the landing of insects carried in an airstream follows convergence of the airstream with a cold front and rain. Records revealed that the dates in spring and the locations at which such winds converged with cold fronts and rain in almost all instances preceded by a few days the dates on which infections of horses with EEEV were notified. Sellers and Maarouf (1990) considered it probable that *Culiseta melanura*, a main vector of EEEV, was transported by wind on those occasions.

45.1.6 History of Eastern equine encephalitis in Massachusetts

Descriptions of outbreaks of disease in horses in three coastal counties of Massachusetts in 1831 showed them to have had the characteristics of the disease known much later as Eastern equine encephalitis. At Middleborough, Maryland, from the recall of one resident, an estimated 75 horses died, while the number that had the disease to a greater or lesser degree and recovered was about 25. Diseased horses inclined their heads to either the left or the right and would walk only in one or other direction, so tending to gyrate in circles. At a more advanced stage of the disease, horses became prostrate and unable to rise. Post-mortem examination revealed inflammation in both lobes of the brain, and in some cases accumulation of fluid in the ventricles of the brain. At that time there was no concept of infective agents, and a commonly proposed treatment was the withdrawal of blood – varying from small volumes to 3 to 4 gallons – to unload congested organs (Phinney, 1831; Thompson, 1831; Peck, 1832).

In 1900–1901, and again in 1912, equine encephalitis outbreaks were prevalent in Maryland and some other eastern states. From July to September 1933, the disease recurred in Virginia, Maryland and Delaware in a very acute and malignant form, with a prevalence rate among horses and mules of 90% or more and a death rate on some farms amounting to 25–30% (Giltner and Shahan, 1933). By that time the infective agent of equine encephalitis was known to be a filterable virus. Strains isolated from both eastern and western cases were highly pathogenic to guinea pigs, less pathogenic to mice, and mildly virulent to rabbits. In contrast, field cases of equine encephalitis in the east were more acute than those in the west, and histopathological studies revealed more severe damage to the CNS of horses in the east. Immunological differences were revealed between the viruses from eastern and western sources. In neutralization experiments in guinea pigs, serum from eastern sources was protective against inoculated virus from eastern sources but not against virus from western sources, and vice versa. There was now sufficient evidence to recognize two distinct encephalitic diseases (Giltner and Shahan, 1933; Ten Broeck and Merrill, 1933).

In 1938, an outbreak of EEE among humans in Massachusetts hospitalized at least 34 people, most of whom died. Komar and Spielman (1994) surmised that environmental changes due to the colonization of North America caused a hiatus in transmission of EEEV for 100 years or more, to be followed by the re-emergence of EEE as a prominent zoonosis. To explore this hypothesis, they examined the effects of changes in the landscape of Massachusetts on the occurrence of the vertebrate hosts of EEEV and of its vector, and the known

history of EEE in that state. When the first European colonists arrived, north-eastern America was extensively forested. By the mid-1880s, exploitation of forests for timber and their transformation for agricultural use had stripped virtually the entire Massachusetts countryside of its forest cover. The demand for cedar wood, for house building and other uses, led to the destruction of the forested cedar swamps. During much of the 20th century, wetlands were viewed as undesirable; coastal salt marshes were drained to suppress mosquito populations, and freshwater marshes were 'reclaimed' for cultivation. Under pressure of the growing human population, urban and suburban developments extended into areas of forested wetland.

The disappearance of forest, and other human influences, led to marked changes in the avian fauna, notably the disappearance of birds such as the snowy egret (*Egretta thula*) and glossy ibis (*Plegadis falcinellus*) that colonize wetland habitats. In contrast, American robins (*Turdus migratorius*), which roost colonially in swamps, exploited the changing landscape and thrived. Loss of habitat probably rendered *Culiseta melanura* a rare species in the 19th and early 20th centuries. Because eastern North America was largely deforested by the mid-1800s, few trees were mature enough to become buttressed or windthrown until the mid-1900s. Dyar (1922) described *Cs. melanura* as rare.

From the 1920s, the need to preserve the forests as a sustainable resource was perceived, and the forestry industry refrained from felling large tracts of forest. From the mid-1960s onwards, the conservation movement acquired legal force for the protection and renewal of wetlands in Massachusetts. Now, mature trees cover much of the region. Wetland conservation promoted mosquito abundance, particularly that of *Cs. melanura*, which is now one of the most abundant mosquitoes in freshwater swamps. It also provided habitats for many bird species, including waders and other amplifying hosts of EEEV. Of the dead-end hosts of EEEV, horse populations declined, whereas human populations grew. Komar and Spielman (1994) concluded that landscape and faunal changes produced a hiatus in the transmission of EEEV that lasted almost 100 years, with zoonotic EEE gradually emerging again in Massachusetts during the 20th century.

45.1.7 Transmission in the Neotropical Region

Eastern equine encephalitis virus occurs in the Caribbean, in Central America, and in South America as far south as Argentina. In the Caribbean, EEEV is represented by strains of lineage I, the range of which extends southwards from North America. Lineage II is present in Central America, and also in South America where it is known principally from Brazil. Lineage III extends across the northern part of the South American continent, while lineage IV is known from a single location in Brazil (Figure 45.1) (Brault *et al.*, 1999; Weaver, 2001). The EEEV lineages present in Central and South America are less virulent in dead-end hosts than lineage I in North America. They occasionally cause illness and death in horses, but human infections are rare and seldom result in overt disease (Scott and Weaver, 1989).

Enzootic transmission of EEEV in Central and South America occurs almost continuously through the year, but details of the transmission cycle are poorly known. In South America, enzootic transmission often occurs in remote rainforests. Most isolations of the virus from mosquitoes have been from species of *Culex* (*Melanoconion*), notably *Cx. taeniopus* in Panama and Brazil, *Cx. dunni* and *Cx. panocossa* in Venezuela, and *Cx. pedroi* in Peru (Shope *et al.*, 1966; Srihongse and Galindo, 1967; Walder *et al.*, 1984; O'Guinn *et al.*, 2004). Wild-caught *Cx. pedroi* became infected after feeding on animals with a viraemia of $\approx 10^5$ PFU ml^{-1} of lineage II or III EEEV (Turell *et al.*, 2008b). The larval habitats of species of *Melanoconion* are typically swamps, marshes, pools and the edges of slow-moving rivers. The habitats may be heavily shaded or open to the sun.

The identities of vertebrate hosts of EEEV in Central and South America have been established by serodiagnosis. During a 2-year study undertaken near Belém, Pará, Brazil, in a forest where EEEV

was enzootic, and in adjacent 'open field', 2068 birds were bled and screened. Almost all plasmas positive for anti-EEEV antibodies were from birds captured in forest (both dry and inundated primary forest and secondary forest) or from forest-nesting birds. The mean positivity rate was 2.9% in 1963 and 1.4% in 1964. In 1963, among sera samples collected in forest from 448 rodents of three species, and 148 marsupials of five species, only one sample, from an arboreal marsupial, was positive. In 1964, no positive sera were obtained from 162 rodents and 49 marsupials. EEEV was isolated from sentinel chicken, sentinel mice and a sentinel *Cebus* monkey exposed in the forest, and it was also isolated from five pools of *Cx. taeniopus*, the putative vector, which constituted 25% of mosquitoes responding to chicken bait (Shope *et al.*, 1966). EEEV was isolated from sentinel hamsters exposed in tropical forest in northern Brazil at a location where members of an indigenous human population proved seropositive (Dickerman *et al.*, 1980).

In an area of Venezuela with extensive swamps, where EEEV was isolated from *Cx. panocossa* and *Cx. dunni*, 99 mammals and reptiles were captured and bled for serological tests. EEEV-specific antibodies were present in four out of 54 opossums (*Didelphis marsupialis*), but absent from nine spiny rats (*Proechimys guairae*) and two edentates. Among 34 reptiles tested, two out of seven lizards (*Tupinanbis nigropunctatus*) were seropositive, as were one out of three turtles (*Podocnemis cayennensis*) (Walder *et al.*, 1984).

The responsiveness to marsupials and rodents shown by foraging females of certain species of *Culex* (*Melanoconion*) that are putative vectors of EEEV has been treated by some authors as circumstantial evidence for the identification of those orders of mammals as hosts of EEEV. In Panama, rodents were the preferred host of *Cx. taeniopus*, but the females frequently attacked birds or humans (Srihongse and Galindo, 1967). In Trinidad, *Cx. taeniopus* responded strongly to marsupials, and less strongly to rodents or birds. The 'engorgement indices' for two opossums (*Didelphis marsupialis* and *Caluromys philander*), a squirrel (*Sciurus granatensis*) and a dove (*Leptotila rufaxilla*) were 10.99, 7.22, 3.55 and 0.45, respectively (Davies, 1978).

The evidence, in total, indicates that certain bird species are natural hosts of EEEV, and that certain marsupials may be natural hosts. Sentinel rodents can become infected with EEEV, but no evidence has been produced implicating wild rodents.

45.1.8 Summary

(i) In eastern and south-eastern North America, Eastern equine encephalitis is enzootic in many forested freshwater swamps where EEEV is transmitted between birds and mosquitoes in an enzootic cycle.
(ii) Over the greater part of the range of EEEV in North America, *Culiseta melanura* is the sole enzootic vector. In central Alabama, *Culex* (*Mel.*) *erraticus* is a putative enzootic vector. Infections with EEEV in mosquitoes that feed predominantly on reptiles or amphibians merit further investigation.
(iii) Restriction of the enzootic cycle to swamp habitats is due to the tight habitat requirements of *Cs. melanura* larvae, the host preference of the adult females of *Cs. melanura* for birds, and the limited dispersion of the adults by flight.
(iv) In northern swamps, viraemic birds may be found in May and June, before the first isolation of EEEV from *Cs. melanura*. In spring and early summer, EEEV is amplified in birds that hatched earlier in the same year. The species that, early in the year, possess anti-EEEV antibodies are permanent residents or summer residents of the swamps. These birds had hatched one or more years previously and, presumably, had been infected in a previous year.
(v) The source of the EEEV that first infects susceptible birds or mosquitoes in enzootic foci during the spring or early summer is not known.
(vi) Epornithics are thought to be initiated when infective *Cs. melanura* transmit EEEV to birds at short distances from swamps, when other mosquito species feed on infective birds, and the number of infective vectors is greatly increased. Putative epornithic vectors include *Cq. perturbans*, *Oc.*

canadensis, *Oc. sollicitans*, *Cx. erraticus*, *Cx. salinarius* and *Am. vexans*. Probably, certain of those mosquitoes act as bridge vectors and transmit EEEV to dead-end hosts.

(vii) Circumstantial evidence suggests that in the spring EEEV is carried northwards by winds transporting infected females of *Cs. melanura*.

(viii) Relatively little is known of the transmission cycles of EEEV in Central and South America. The amplifying hosts are thought to be principally forest birds. The known mosquito hosts and putative vectors are species of *Culex* (*Melanoconion*).

45.2 TRANSMISSION OF DENGUE VIRUSES

Dengue viruses are perpetuated in transmission cycles that involve aedine mosquitoes and primate hosts. Sylvatic cycles involving monkeys and mosquitoes that exist in forest canopies probably preceded transmission to humans. At some time, humans became amplifying hosts, and now quite separate transmission cycles exist that involve only humans and synanthropic mosquitoes. For many years, human infections were recognized as dengue fever (DF), a non-fatal disease characterized by fever, muscle and joint pains, and rash. A more serious form of the disease was first recognized in 1953 in the Philippines, and is now known as dengue haemorrhagic fever (DHF). Major clinical features of DHF include high fever, haemorrhage, an enlarged liver and, in severe cases, signs of circulatory failure. In seriously affected patients, plasma leakage may lead to a substantial decrease in blood volume producing dengue shock syndrome (DSS), which can be fatal. The term 'dengue' refers to the entire spectrum of dengue viral disease (WHO, 1997). The clinical characteristics of dengue, in its different manifestations, are described in Section 44.10.3.b.

In recent centuries, changes in the distributions of dengue viruses and of their main vector (*Stegomyia aegypti*) were associated with pandemics of dengue fever. During the last 30 years of the 20th century, a dramatic increase occurred in geographical spread, number of cases and severity of the disease owing to three causes: (i) changes in human demography, notably uncontrolled population growth and urbanization in the absence of appropriate water management; (ii) circum-global spread of dengue serotypes and of the main vector, through the agencies of human travel and trade; and (iii) erosion of vector control programmes. Dengue virus is now present in almost every country between the tropic of Capricorn and the tropic of Cancer. During the 1960s and 1970s, in countries where dengue virus was endemic, the occurrence of DHF/DSS extended from its primary location in major cities to smaller cities and towns. Seasonal and cyclical epidemics became established, with large outbreaks occurring at 2–3 year intervals (Kuno, 1997; Gubler, 1998; WHO, 2000a). In authoritative assessments, dengue was named as the most important mosquito-borne viral disease of humans, with 2.5 billion of the world's population at risk, primarily in tropical developing countries. It was estimated that 50 million cases of the disease occur annually, including hundreds of thousands of dengue haemorrhagic fever, which in many countries is a leading cause of childhood hospitalization and death (WHO, 2001, 2009). Currently, there is no licensed vaccine for dengue, and patients can be given only supportive care. Because a previous infection by one dengue strain provides no protection against infection by another of the other strains, and because of the possibility that immune enhancement by a monotypic antibody might lead to DHF with subsequent natural infections, control of dengue requires an efficient tetravalent vaccine (WHO, 2009). Currently, protection from the disease depends entirely on vector control.

45.2.1 Dengue virus group

Dengue virus is a species of the genus *Flavivirus* and the family *Flaviviridae*. The Dengue virus group is one of the clusters of flaviviruses, originally characterized serologically, that are associated with aedine mosquitoes and primates as hosts, and that cause haemorrhagic disease in seriously affected

primate hosts (Section 44.1.2.b). The group comprises two viruses: *Dengue virus* and *Kedougou virus*, the former consisting of four serotypes or strains. In the Eighth Report of the ICTV (Fauquet *et al.*, 2005) the members of the Dengue virus group were given the following decimal codes, names and acronyms:

Family	00.026 = *Flaviviridae*
Genus	00.026.0.01 = *Flavivirus*
Dengue virus group	
Species	00.026.0.01.013 = *Dengue virus*
Strain	00.026.0.01.013.08.001 = Dengue virus 1 DENV-1
	00.026.0.01.013.08.202 = Dengue virus 2 DENV-2
	00.026.0.01.013.08.203 = Dengue virus 3 DENV-3
	00.026.0.01.013.08.204 = Dengue virus 4 DENV-4
Species	00.026.0.01.023 = *Kedougou virus*
Strain	00.026.0.01.023.08.201 = Kedougou virus KEDV

Much of the serology that led to the distinguishing of four DENV serotypes was based on differences in amino acid sequences of the envelope glycoprotein. Comparison of the complete genomic sequences of the four serotypes produced the same grouping (Westaway and Blok, 1997). For many years the four subspecific taxa of *Dengue virus* (DENV-1, DENV-2, DENV-3 and DENV-4) were classed as 'serotypes', but in the Eighth ICTV Report they were ranked as 'strains'. However, authors still use the term serotype for them, possibly because each serotype comprises a number of geographically separated and genetically distinct strains. Thus, analysis of the nucleotide sequences of the envelope (E)-protein gene in isolates of DENV-2 from 17 locations in nine countries showed them to represent 15 distinct 'strains' (Cologna *et al.*, 2005). To conform to current practice, the taxa DENV-1, DENV-2, DENV-3 and DENV-4 are described as serotypes in this volume.

Microevolution continues within each serotype, and new lineages arise which differ in virulence. Complete sequencing of the E-protein gene from 66 dengue virus strains revealed the phylogeny of the four serotypes and of lineages within them. During the evolution of dengue virus, DENV-4 diverged first, followed by DENV-2, and finally by DENV-1 and DENV-3 (Zanotto *et al.*, 1996). Wang *et al.* (2000) reported similar findings (Figure 45.8). Genetic studies suggest that all four serotypes evolved independently from ancestral, sylvatic viruses and became ecologically distinct. The independent evolution of the serotypes was accompanied by the expansion of the sylvatic progenitor's host range in Asia to new vectors and mammal hosts, probably over a period of several 100 years (Vasilakis and Weaver, 2008, review).

The evolutionary relationships between the sylvatic and so-called 'endemic' lineages of DENV serotypes are also of interest. The sylvatic forms are transmitted in cycles that involve canopy-dwelling primates and forest mosquitoes. The 'endemic' forms (here termed 'rural/urban') are transmitted in rural or urban cycles that involve humans and, mainly, *Stegomyia aegypti*. Strains of both sylvatic and rural/urban lineages were available for each of the serotypes DENV-1, DENV-2 and DENV-4, but only rural/urban strains were available for DENV-3. However, the reported seroconversion for DENV-3 in a sentinel leaf monkey in forest canopy in Malaysia (Rudnick, 1983) indicated that a sylvatic DENV-3 cycle exists there.

Analysis of E-protein gene nucleotide sequences was undertaken with sylvatic strains of DENV isolated from forest mosquitoes or monkeys in Malaysia or Africa, and for rural/urban strains isolated from humans or *St. aegypti* from several countries. The sylvatic isolates from Malaysia and Africa differed genetically from one another, and were genetically distinct from all of the rural/urban isolates. Phylogenetic trees placed sylvatic isolates from Malaysia or Africa at the basal positions of the clades for DENV-1, DENV-2, DENV-4 (Figure 45.8). The presence in Malaysia of the sylvatic forms of all four DENV serotypes, compared with the presence in Africa of sylvatic strains of only DENV-2, suggested that a sylvatic ancestor of all dengue viruses arose in the Oriental Region where it diverged into the four serotypes known today. It was surmised that a sylvatic progenitor evolved into

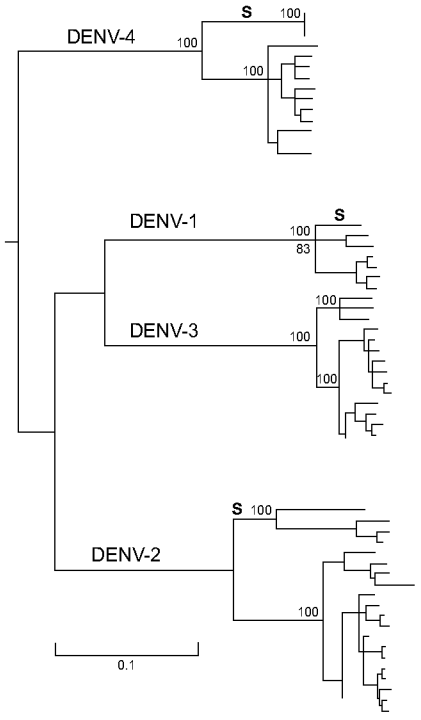

Figure 45.8 Phylogenetic tree derived from the nucleotide sequences of the envelope protein (*E*) gene from sylvatic or rural/urban strains of the dengue viruses (DENV 1–4). The tree shows divergence of the four serotypes and, for three of the serotypes, the divergence of rural/urban strains from sylvatic strains. No sylvatic strains of DENV-3 were available for analysis. (After Wang *et al.*, 2000, but much simplified.) The original tree contained branches for 79 rural/urban strains (here reduced to 46) and eight sylvatic strains. The numbers on branches are bootstrap values that show the percentage support for the hypothesis that the lineages springing from a branch form a monophyletic cluster. Branches labelled **S** represent sylvatic lineages. All other branches represent rural/urban lineages. The scale shows a genetic distance of 0.1 or 10% nucleotide sequence divergence.

the four DENV serotypes and that, later, rural/urban forms evolved independently from each of the four sylvatic lineages. The dates of divergence between the sylvatic and rural/urban forms for three of the serotypes were estimated to be: DENV-2, some 1000 ± 500 years ago; DENV-4, some 600 ± 300 years ago; and DENV-1, 200 ± 100 years ago. The DENV-2 sylvatic form appears to have diverged into Malaysian and African sylvatic lineages some 800 ± 400 years ago. The events leading to the emergence of rural/urban forms from the sylvatic progenitors probably included a change of vectors – from sylvan, canopy-dwelling aedine mosquitoes to *Stegomyia albopicta*, and later to *St. aegypti*. Adaptation to new vectors and new vertebrate hosts must have occurred repeatedly (Wang *et al.*, 2000). From comparison with the rates of substitution that had been estimated for tick- and mosquito-borne flaviviruses, Zanotto *et al.* (1996) concluded that the last two centuries were a period of intense cladogenesis in the dengue serotypes.

Analyses of isolates of strains of DENV serotypes from around the world revealed that their evolution has been a continuing process. Many isolates fell into clusters that were termed topotypes or genotypes, depending on the means of analysis (Westaway and Blok, 1997). Sequencing a fragment from the E/NS1 junction of 40 isolates of DENV-2 from many countries revealed five clusters of isolates which had no more than 6% sequence divergence; these were classed as 'genotypes' (Rico-Hesse, 1990). Phylogenetic analyses based on partial E/NS1 sequences or complete E nucleotide sequences revealed that DENV-1 and DENV-2 each consist of five genotypes, whereas DENV-3 and DENV-2 each consist of four genotypes. The names given to the five DENV-2 genotypes, with their countries of origin, are detailed in Table 45.4.

Strains of individual DENV-2 genotypes have been found circulating in countries in which they were previously unknown, and competing with the local genotypes. Strains of the Cosmopolitan genotype appeared in the Philippines in 1998, and gradually and effectively replaced the existing Asian genotype 2 strains (Salda *et al.*, 2005). During the last two decades of the 20th century, strains of the SE Asian genotype of DENV-2 appeared in the western hemisphere, replacing strains of the American genotype. Viruses of the American genotype were isolated only from patients with dengue fever, and the origin and spread of dengue haemorrhagic fever in the western hemisphere could be linked to viruses of the SE Asian genotype. In the laboratory, following oral intake of virus by mosquitoes, strains of the SE Asian

Table 45.4 Sources of the strains used to define the five genotypes of Dengue virus 2. (From the data of Vasilakis and Weaver, 2008.)

Genotype number	*Genotype name*	*Sources of strains*
I	Asian	
	Asian genotype 1	Malaysia, Thailand
	Asian genotype 2	Vietnam, China, Taiwan, Sri Lanka, Philippines
II	Cosmopolitan	(including) Australia, East and West Africa, Pacific Islands, Indian Ocean Islands, Indian subcontinent, Middle East
III	American	Latin America and older strains collected from the Caribbean, Indian subcontinent and Pacific Islands in the 1950s and 1960s
IV	South-east Asian/American	Thailand, Vietnam/Americas from 1981
V	Sylvatic	Forest mosquitoes, sentinel monkeys or humans in West Africa and South-east Asia

genotype tended to infect two New World strains of *St. aegypti* more efficiently than did strains of the American genotype, but there was substantial within-genotype variation (Rico-Hesse, 1990; Armstrong and Rico-Hesse, 2003). When cultured in human dendritic cells, viruses of the SE Asian genotype replicated more efficiently than viruses of the American genotype (Cologna *et al.*, 2005). When females of a south Texan population of *St. aegypti* were orally infected with DENV-2 of either the SE Asian or American genotype, viral replication in the midgut was significantly greater with the SE Asian virus. The SE Asian virus appeared in the mosquitoes' salivary glands 7 days earlier than the American virus, which resulted in an estimated 2–65-fold increase in vectorial capacity of mosquitoes for the viruses that can cause dengue haemorrhagic fever (Anderson and Rico-Hesse, 2006).

45.2.2 Dispersion of *Stegomyia aegypti* in historical time

(a) Stegomyia aegypti *in Africa*

Stegomyia aegypti arose in Africa, where two subspecies emerged. The synanthropic nature of certain forms of these subspecies is thought to be a derived trait, not present in the ancestral stock.

Stegomyia aegypti formosa occurs in two genetically distinct forms. (i) Sylvan form. Characteristically inhabits tropical forest, where the larvae develop in tree holes and in rock pools formed when rivers dry up during drought. The adults do not respond to humans as hosts. (ii) Peridomestic form. The larvae develop in natural and artificial containers outdoors near human dwellings; the adults attack humans. Genetic analysis of populations showed *St. a. formosa* to be present in Africa south of the Sahara: the sylvan form was present in East Africa; the peridomestic form in West Africa. Just outside the African continent, *St. a. formosa* is present in Madagascar and on two small, nearby islands – Europa Island and La Réunion.

Stegomyia aegypti aegypti is synanthropic, and often is described as domestic. The larvae develop in clean water in human water-storage vessels; the adults rest and blood-feed indoors. Genetic analysis of populations showed *St. a. aegypti* to be present in East Africa but absent from West Africa (Haddow, 1945a; Garnham *et al.*, 1946; Mattingly, 1957; Powell *et* al., 1980; Ballinger-Crabtree *et al.*, 1992; Failloux *et al.*, 2002).

(b) Dispersion to the New World

During relatively recent historical time, *St. aegypti* dispersed widely beyond Africa, being transported

on ships. During long voyages the females would have been able to obtain human blood, and the presence of bodies of fresh water would have permitted development of the aquatic stages. The first scientific record of *St. aegypti* in the New World was that of Fabricius (1805), who described it from the Antilles (West Indies) under the name of *Culex fasciata*. However, *St. aegypti* could have reached the Americas much earlier if it had been transported in ships: there was commerce between West Africa and the Caribbean during the 16th century, and an active slave trade from early in the 17th century. The occurrence in named localities of dengue fever or yellow fever provides strong circumstantial evidence for the presence there of *St. aegypti*. There is evidence of dengue fever in the Lesser Antilles during the 17th century (Siler *et al.*, 1926), and of yellow fever occurring more widely in coastal cities on the eastern seaboard of the Americas in the 17th century (Monath, 1989).

Genetic analyses undertaken during the 1960s to 1980s showed that populations with the genetic characteristics of *St. a. aegypti* from Africa were widely distributed in tropical, subtropical and warm-temperate parts of the Americas, from Brazil to Mexico and the Caribbean to most of the USA. Such populations of *St. a. aegypti* were absent from the south-eastern USA, where populations of *St. a. formosa* were found (Tabachnick and Powell, 1979; Tabachnick *et al.*, 1979; Powell *et al.*, 1980; Wallis *et al.*, 1983, 1984; Tabachnick, 1991). Reviewing many published reports, Bracco *et al.* (2007) concluded that the data supported the hypothesis that *St. a. aegypti* was introduced into the Americas during the 17th and 18th centuries, and experienced a bottleneck caused by vector control measures adopted during the 1950s and 1960s.

(c) Dispersion to Asia

Macdonald (1956) stated that there was a record of the presence of *St. aegypti* in South-east Asia before 1860, citing Walker's (1859) article 'Catalogue of the dipterous insects collected at Makessar in Celebes by Mr A.R. Wallace, with descriptions of new species'. One of the new species was named *Culex impatibilis*, the description being of a male specimen. Edwards (1932) treated *Culex impatibilis* Walker as a junior synonym of *Aedes* (*Stegomyia*) *aegypti* L. Knight and Hull (1952) commented that the specimen, in the Walker Collection (Natural History Museum, London), was then in very poor condition and was female not male (not unknown among Walker's species). They identified it as a member of the 'Albolineatus Group' and keyed it to *Aedes* (*Ste.*) *hoogstraali*, from which its condition did not permit further differentiation. Reinert (1985) resurrected the subgenus *Scutomyia* for the Albolineatus Group of *Aedes*, and subgenus *Scutomyia* was restored to generic rank by Reinert *et al.* (2004), the genus including *Sc. impatibilis* (Walker). The mosquito sent by Wallace from Makessar was aedine but not *St. aegypti*.

By 1908, in Malaya (now the Malaysian peninsula), *St. aegypti* was largely a mosquito of ports, but it slowly spread from the coast, displacing *St. albopicta* in some locations. A preliminary survey undertaken during 1952 suggested that *St. aegypti* was abundant in coastal towns and in kampongs well outside them, but that further inland it was limited to towns and present at much lower densities (Reid, 1954). During 1954–1955, 'house indices' were recorded in 49 towns and villages (being the percentage of the houses and surrounding compounds that had *St. aegypti* larvae in at least some containers). The house indices were: coastal towns, 38%; coastal villages, 32%; inland towns, 34%; inland villages, 18%. In four inland villages no *St. aegypti* larvae were found. Drums and earthenware jars used for water storage contributed up to one-half of the aquatic habitats of *St. aegypti*. All junks that sailed up and down the coast carried one or two jars of fresh water, and most jars contained *St. aegypti* larvae (Macdonald, 1956). In 1974, a survey of 157 towns showed that *St. aegypti* was present in all inland locations surveyed earlier, and that in some of them it had rather high house indices (Cheong, 1986).

(d) Eradication attempts in the Americas

During the 20th century, control operations against *St. aegypti* were undertaken in many tropical countries to reduce or eliminate the risk of

epidemics of yellow fever or dengue. The history of these undertakings reveals the key role of *St. aegypti* in urban transmission cycles of those two viruses. Most significant were operations undertaken in the Caribbean and in Central and South America. Under the leadership of W.C. Gorgas, and through the reduction of *St. aegypti* populations, yellow fever was eliminated from Cuba in 1901 and from the canal zone of Panama by 1906. Between 1915 and 1925, with Gorgas now heading the Rockefeller Foundation's Yellow Fever Commission, yellow fever virus was eradicated from Ecuador, Peru, Colombia and the countries of Central America, and progress had been made in Brazil. The proposal in 1934 that *St. aegypti* be eradicated from Brazil was received with incredulity, but a campaign was initiated and by 1940 it had indeed been eradicated. In 1947, a programme to eradicate *St. aegypti* from the western hemisphere for the elimination of the threat of urban yellow fever was started by the Pan American Sanitary Bureau. By the late 1960s, this mosquito had been eradicated from most New World countries that it had invaded, but not from Suriname, Guyana, Venezuela, the southern USA, or some Caribbean islands (Lourenço-de-Oliveira *et al.*, 2004). Urban yellow fever and dengue disappeared from wherever *St. aegypti* had been eradicated (Soper, 1963; Spielman and D'Antonio, 2001).

During the 1960s, opposing views were expressed by two individuals on the eradication of *St. aegypti* from the western hemisphere. Dr F.L. Soper, who, with his associates, had eradicated *Anopheles gambiae* from north-east Brazil, and had eradicated *St. aegypti* from cities and large rural areas of northern and eastern Brazil (Andrews and Langmuir, 1963), insisted on the importance of completing the well-advanced programme of *St. aegypti* eradication (Soper, 1963). Dr D.J. Sencer, Director of CDC, recommended that the eradication programme be replaced by other methods of disease control (Sencer, 1969). In the early 1970s, the eradication programme was ended without having reached its goal. Subsequently, *St. aegypti* reinvaded many countries from which it had been eradicated; by the 1980s it had reinvaded Central and South America, and by 1995 it had become re-established in all countries of the hemisphere except Canada, Bermuda, Chile and Uruguay. As noted earlier, from 1977 additional DENV serotypes arrived in the Americas, and in less than 20 years the American tropics acquired a serious dengue/DHF problem. There have been no major urban epidemics of yellow fever in continental South America since 1942, but during the period 1985–1999 WHO received reports of 2894 cases of sylvatic yellow fever (Monath *et al.*, 1981; Gubler, 1997; Gratz, 1999). It is difficult to assess how far the Americas could have been kept free of *St. aegypti* had it once been eradicated.

45.2.3 Dispersion of dengue virus in historical time

Only since the mid-20th century has virological diagnosis of dengue fever been possible. Identification of the aetiological agents responsible for historical outbreaks of dengue fever relies on descriptions of symptoms, but the clinical symptoms of dengue fever can be indistinguishable from those of some other viral infections (George and Lum, 1997). Even so, when the clinical symptoms of an early outbreak were described, and the possible presence of other viruses at that location and that time can be surmised, epidemiologists have identified historical outbreaks of dengue fever with some confidence. Smith (1956) examined records of outbreaks of dengue-like illness from India and some South-east Asian countries for the period 1779–1954, and described a possible history of dengue in South and South-east Asia (Figure 45.9). His conclusions were partly speculative but merit recall.

The evidence indicated that dengue virus originated in tropical Asia, and dispersed from that region over a long but uncertain period of time. Each territory of South and South-east Asia had native to it at least one species of the Scutellaris Complex of *Stegomyia* (Section 45.2.4.a) that was an effective vector of DENV. Smith (1956) suggested that for centuries these species had been the vectors

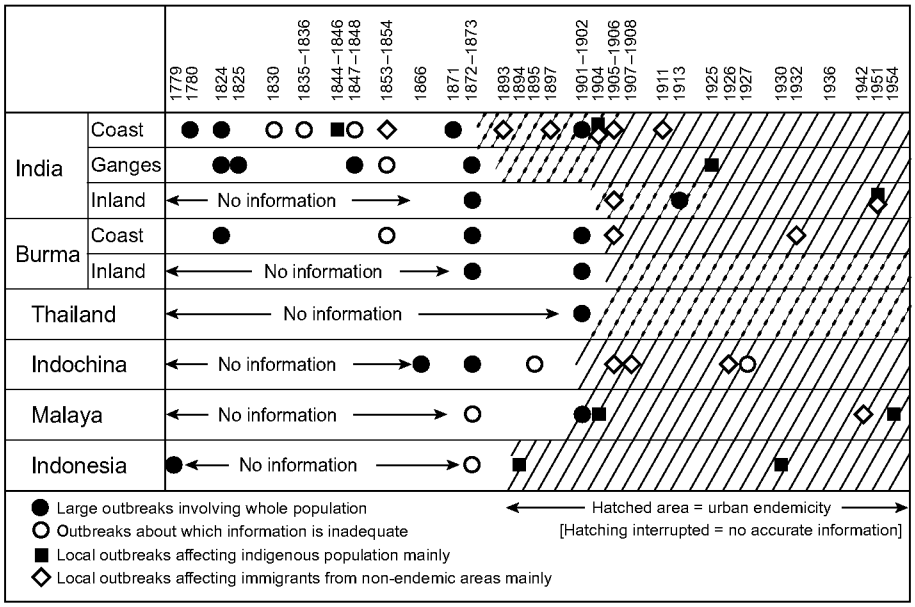

Figure 45.9 Records of outbreaks of dengue or dengue-like illness in tropical Asia during the period 1779 to 1954. (After Smith, 1956.) Burma is now Myanmar; Indochina is now Vietnam; Malaya is now Peninsular Malaysia.

of rural dengue in this region, causing a steady rate of transmission among rural people and raising their immunity, so that only sporadic cases and local outbreaks occurred. Town dwellers had less immunity and were subject to periodic epidemics.

From limited distribution records, Smith (1956) concluded that *St. aegypti* invaded South-east and South Asia in strength during the second half of the 19th century, first becoming established in seaports and later gradually spreading inland, mainly along rivers and other routes of communication. Large urban epidemics recurred in India and some South-east Asian countries until about the end of the 19th century. By that time, dengue fever had become endemic in the towns and cities of these regions and, consequently, their urban populations had increased immunity and urban epidemics became rare. During the first half of the 20th century small outbreaks occurred in the indigenous populations of a number of countries in South and South-east Asia, while epidemics of varying size affected immigrants from non-endemic areas. This could be explained by the distribution of vectors and the immune status of human populations.

The reappearance of dengue fever in a country long after the first arrival there of dengue virus probably reflected either a general loss of acquired immunity or the dispersion of a different serotype of DENV or a different strain. During the period 1900–1902, epidemics of dengue fever occurred in ports and coastal towns in South-east and East Asia. This series of outbreaks was thought to have started in Java, and to have advanced north-west to Singapore (where dengue fever reappeared after an absence of 30 years), Penang and Bangkok, northwards to Hong Kong, and later to other coastal towns in China and to Japan. In Penang, a large proportion of the indigenous population suffered from dengue fever as well as many Europeans (Skae, 1902; Rudnick, 1986a, review).

The wider dispersion of dengue virus and the development of dengue as a global health problem were described in Gubler (1997). A pandemic of dengue fever and dengue haemorrhagic fever occurred in the Asian and Pacific theatres during

and after World War II, associated with ecological changes and huge movements of people. The physical destruction from warfare had created innumerable artificial habitats for mosquito larvae, as did the increased need for storage of domestic water, while the movements of hundreds of thousands of troops transported *St. aegypti* widely and provided susceptible individuals for epidemic transmission. Post-war, the Asian and Pacific regions experienced different outcomes.

In the years following World War II the urbanization of South-east Asia began, but water management procedures were inadequate or faulty and the populations of *St. aegypti* grew. Dengue virus, often as all four serotypes, was maintained in human–*St. aegypti* cycles in most urban centres. The demographic changes led to epidemics of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) in South-east Asia, and within a few years to a pandemic. The emergence of epidemic DHF in the 1950s was followed by the recurrence of epidemics every 3 to 5 years in most South-east Asian countries. The 1980s saw a dramatic expansion of epidemic DHF, which moved west into India and Sri Lanka, and north-east into southern China (Figure 45.10). By 1995, DHF was the principal cause of hospitalization and death among children in many Asian countries.

Between 1942 and 1945, due to the disruptive effects of war, a major pandemic caused by DENV-1 occurred over most of the Pacific region, but for some 20 years after the war dengue virus had disappeared from the region. Between 1964 and 1979 all four serotypes appeared, and epidemics spread through the Pacific islands. Epidemics resulting from DENV-2 on four island groups provided the first field evidence that the virulence of a dengue serotype could change as it moved through human populations. On Tahiti in 1971, and in New Caledonia in 1972, the prevalence rate was >75%, the disease was severe and the outbreaks were 'explosive'. On American Samoa in 1972, and in Tonga in 1974, the prevalence rates were 57% and 35%, respectively; the disease was mild, and the outbreaks were 'smouldering' and silent, respectively. It is uncertain when dengue virus reached Africa, but the divergence of the Malaysian and African lineages of DENV-2 some 800 ± 400 years ago (Section 45.2.1) places that event well before all historical records. Even during the 20th century, surveillance for dengue in Africa was poor.

Figure 45.10 Extension of the geographical distribution of epidemic dengue haemorrhagic fever in Asia, 1950 to 1996, by period and country. (From Gubler, 1997.) These maps conform to the practice of medical-map makers whereby infections are shown as occurring throughout whole countries when, at the time indicated, they may have been present in only a part.

Reports of epidemic dengue fever from East, West and Southern Africa increased greatly after 1980.

Epidemic dengue fever occurred in Caribbean countries during World War II, but from 1946 to 1963 there was no recorded epidemic although DENV-2 was endemic in the region. Only after another 15 years did epidemic dengue re-emerge as a major health problem in the Americas. The period of quiescence was probably due to the *St. aegypti* eradication programme (Section 45.2.2.d), which was successful in most countries in the Western Hemisphere. The stopping of the programme in the early 1970s led to progressive reinfestation of the region with *St. aegypti*, while from 1977 onwards additional DENV serotypes arrived in the Americas, and new strains arose, resulting first in cases of uncomplicated dengue fever and later in major epidemics of DHF/DSS. In less than 20 years, the situation in the American tropics changed from an absence of dengue to a serious dengue/DHF problem.

45.2.4 Natural host ranges

(a) Mosquito hosts and vectors

The mosquitoes that are known to be natural hosts of dengue virus, and that are proven or putative vectors, belong to three aedine genera:

Subfamily Culicinae; Tribe Aedini
Diceromyia
Furcifer Group: *Di. furcifer*, *Di. taylori*
Downsiomyia: *Do. pseudonivea*, *Do. subnivea*
Stegomyia
Aegypti Group: *St. aegypti aegypti*, *St. aegypti formosa*
Africana Group: *St. africana*, *St. luteocephala*, *St. neoafricana*, *St. opok*
Scutellaris Group
Albopicta Subgroup: *St. albopicta*
Scutellaris Subgroup: *St. scutellaris*, *St. polynesiensis*, *St. cooki*, *St. hebridea*.

Systematics. *Downsiomyia*: Vargas (1950); Knight and Marks (1952); Colless (1958, 1959); Harrison *et al.* (1991); Reinert and Harbach (2006). *Stegomyia*: Huang (1979a, 1990, 2004); Huang and Hitchcock (1980). *Diceromyia*: Ferrara *et al.* (1984); Hervy *et al.* (1985); Huang (1986b); Cook *et al.* (2005). Aedine genera: Reinert *et al.* (2004, 2009). **Implication as vectors.** *Diceromyia*: Cordellier *et al.* (1983); Diallo *et al.* (2005b). *Downsiomyia*: Rudnick (1983). *Stegomyia*: Cordellier *et al.* (1983); Rudnick (1983); Hervy *et al.* (1984); Rodhain and Rosen (1997); Chung *et al.* (2001); Chung and Pang (2002); Diallo *et al.* (2005b).

Downsiomyia was named as a subgenus of *Aedes* by Vargas (1950) for species formerly assigned to the Niveus Subgroup (of the Geniculatus Group) of *Aedes* (*Finlaya*), and was elevated to generic rank by Reinert *et al.* (2004). The use by some authors of the name *Aedes niveus* (now *Downsiomyia nivea*) was incorrect; Harrison *et al.* (1991) stated that *Ae. niveus* was known only from the Philippines. Characteristically, species of the genera *Stegomyia*, *Diceromyia* and *Downsiomyia* have desiccation-resistant eggs, develop in tree holes, and bite outdoors. *Stegomyia a. aegypti* is strongly synanthropic and its domestic form bites indoors.

(b) Vertebrate hosts

In forests of South and South-east Asia and West Africa, monkey populations function as amplifying hosts of dengue virus. Infected monkeys, apparently, do not suffer severe pathological effects. A report from Sri Lanka of an outbreak due to DENV-2 in a population of toque macaques revealed something of the epizootiology of sylvatic dengue fever. Within a 3 km^2 area, some 94% of the macaque population became infected, and for the following 8 years, at least, there was no further transmission in that area (de Silva *et al.*, 1999). Humans are also amplifying hosts. Whether, in nature, dengue viruses infect only primates is uncertain. Claims that some wild bats were seropositive for dengue viruses (Platt *et al.*, 2000, 2001) were challenged by Scott (2001), but, even if seropositivity in bats is confirmed, much more evidence is needed to implicate bats as amplifying hosts. The following primate species have been implicated by serological or virological findings as natural hosts of dengue virus:

Cercopithecidae (Old World monkeys)
Subfamily Cercopithecinae
Erythrocebus: *E. patas* (patas monkey)
Macaca: M. *fascicularis* (syn. M. *irus*; long-tailed macaque), M. *fuscata* (Japanese macaque), M *nemestrina* (pigtail macaque), M. *radiata* (bonnet macaque), M. *sinica* (toque macaque)
Chlorocebus: C. *aethiops* (vervet monkey)
Papio: *P. hamadryas* (hamadryas baboon)
Subfamily Colobinae (leaf monkeys)
Presbytis: *P. melalophos* (mitred leaf monkey)
Trachypithecus: *T. cristatus* (silvered leaf monkey), *T. obscurus* (dusky leaf monkey)
Hominidae
Pongo: *P. pygmaeus* (orangutan)
Homo: *H. sapiens* (human).

Systematics: Wilson and Reeder (2005). **Identification** as natural hosts of dengue virus: Rudnick (1965, 1983, 1986b); Yuwono *et al.* (1984); Saluzzo *et al.* (1986); Rodhain (1991); de Silva *et al.* (1999); Wolfe *et al.* (2001).

45.2.5 Mosquito–virus–human interactions

Experimental studies on the transmission of dengue virus were undertaken in the Philippines with volunteers from the US Army. A latent period of some 4–8 days occurred between infection through the bite of *St. aegypti* and the onset of clinical symptoms; the period of viraemia usually lasted 4–5 days. Patients were most infective to mosquitoes during the first day of perceived illness and viraemia. By the end of the third day of viraemia, the virus was at a low titre or had disappeared from the peripheral circulation. Certain individuals were infective to blood-feeding mosquitoes for 6–18 h before the onset of clinical symptoms. Such pre-symptomatic viraemia is epidemiologically important, because it occurs in individuals who are still active and who may have contact with vectors in other locations. The extrinsic incubation period of 17 days (range 11–20 days) was measured by allowing batches of mosquitoes to feed on viraemic patients and determining the date on which virus was first transmitted to healthy volunteers (Siler *et al.*, 1926). In later years, experimental studies on dengue virus transmission declined. Only rarely was it acceptable to use humans in experiments, and animal models, in the full sense of the term, were not available (Cologna *et al.*, 2005), although transmission to monkeys by bite could be tested. In most experiments on infection of mosquitoes, the mosquitoes were stimulated to feed on suspensions containing dengue virus, but the virus titre needed to infect mosquitoes was several orders of magnitude higher than when mosquitoes fed from a viraemic human (Rodhain and Rosen, 1997; Rothman, 1997).

(a) Mosquito hosts: susceptibility and refractoriness

Wherever in nature a mosquito-borne arbovirus is maintained in transmission cycles, the virus and mosquito populations are exposed to the local selective forces and each of the two populations becomes to some extent adapted to the other. Experimental exposure of a host to an allopatric virus does not precisely mimic the natural situation for either. Also, once taken to a laboratory, viruses and vectors are rapidly altered by passage or colonization. For example, after oral intake of a multi-passaged strain of DENV-2, females of *St. albopicta* that were four generations from the wild (F_4) showed significantly lower susceptibility than F_5 females. Whereas the susceptibility rates of females of a long-colonized strain ($F_{>40}$) of *St. albopicta* were 77.8–96.9%, those of the F_4 females were 5.3–25.0% (Vazeille *et al.*, 2003). In most investigations, the presence of virus in the head some 14 days after oral intake was taken to indicate susceptibility to infection.

A great deal of research has been carried out on the susceptibility of mosquito hosts to infection with DENV-2 and, necessarily, the findings must be summarized.

(i) Susceptibilities of *St. a. aegypti*, *St. a. formosa* and *St. albopicta* to oral infection with DENV-2. Wild-caught (P_w) parental females and their F_1–F_2 offspring, from mostly Old World populations, showed females of *St. a. aegypti* usually to be significantly more susceptible to oral infection

with DENV-2 than females of either *St. a. formosa* or *St. albopicta*. Of populations collected in the South Pacific, South-east Asia, the Indian Ocean, West Africa or South America, all populations of *St. a. aegypti* proved highly susceptible to infection with DENV-2 (infection rates of 70–100%), whereas all populations of *St. a. formosa*, except that from La Réunion, were substantially less susceptible (13–57% infection rates) (Table 45.5) (Failloux *et al.*, 2002). In Madagascar, the susceptibility rates were 25–40% for *St. a. formosa* populations and 33–100% for *St. albopicta* populations (Vazeille *et al.*, 2001). Susceptibility rates in F_1 or F_2 adult females reared from larvae or pupae collected from sympatric aquatic habitats in Vietnam were 93.2–100% for *St. a. aegypti* and 21.2–45.8% for *St. albopicta*. Similar rates were obtained with F_1 or F_2 females that originated from allopatric habitats in Chiang Mai, Thailand: 97.3–98.7% for *St. a. aegypti* and 24.6–28.8% for *St. albopicta* (Vazeille *et al.*, 2003).

(ii) Genetic diversity in geographically separate populations of *St. aegypti* and *St. albopicta* in countries where dengue is endemic, and their susceptibility to infection with DENV-2. Tests were undertaken on wild-caught females (the P_w generation) and/or on females of the earliest filial generations (F_1 and F_2).

Isoenzyme analysis of P_w females of *St. albopicta* showed high genetic diversity among ten Brazilian populations, among nine North American populations, and between the Brazilian and the North American populations. Genetic differentiation increased slightly with geographical distance. Rates

Table 45.5 Susceptibility of adult females of the two subspecies of *Stegomyia aegypti*, collected in different regions of the world, to oral infection with Dengue virus 2 (isolated in Bangkok in 1974). (From the data of Failloux *et al.*, 2002.)

Location	*Batches tested*	*Subspecies*	*Generation*	*Behavioural form*	*Infected (%)*
South Pacific					
French Polynesia[1]	23	*St. aegypti aegypti*	F_1	Domestic	70–100
South America					
French Guiana[2]	25	*St. aegypti aegypti*	F_0	Domestic	78–100
South-east Asia					
Vietnam[3]	19	*St. aegypti aegypti*	F_0	Domestic	88–100
Vietnam[4]	3	*St. aegypti aegypti*	F_0	Domestic	91–96
Cambodia[5]	2	*St. aegypti aegypti*	F_0	Domestic	98–100
Indian Ocean					
La Réunion	1	*St. aegypti formosa*	F_0/F_1	Sylvan	98
Europa Island	1	*St. aegypti formosa*	F_0	Sylvan	13
Madagascar[6]	5	*St. aegypti formosa*	F_1	Sylvan	25–40
West Africa					
Gabon[7]	1	*St. aegypti formosa*	F_0	Sylvan	57
Ivory Coast[8]	2	*St. aegypti formosa*	F_1	Sylvan	20–36

[1] Tahiti and Moorea; [2] Various sites; [3] Ho Chi Minh City; [4] Nha Trang; [5] Phnom Penh; [6] Various sites; [7] Franceville; [8] Bouaké.

The batches of *Stegomyia* were collected as larvae or pupae, mostly from artificial containers, but the batches from La Réunion and Madagascar were collected from natural larval habitats. Populations were identified to subspecies by their isoenzyme characteristics. Susceptibility to infection was determined by feeding 5–7-day-old F_1- to F_3-generation females on an artificial meal containing DENV-2, and 14 days later examining head squashes for virus by indirect immunofluorescence. The artificial meal included washed rabbit erythrocytes, ATP as phagostimulant and DENV-2 at a final titre of $10^{8.2}$ MID_{50} (50% mosquito infective dose) ml^{-1}. The virus strain used had been isolated in 1974 from a human serum sample from Bangkok, Thailand.

of infection with a four-passaged strain of DENV-2 from Bangkok ranged from 22.5% to 89.0% in females of the ten Brazilian strains and from 38.6% and 44.5% in the two North American strains tested (Lourenço-de-Oliveira *et al.*, 2003).

Use of the same methods to examine P_w adults of 23 strains of *St. aegypti* from widely separated locations in Brazil showed that genetic differentiation increased with distance between the sources of the mosquito strains. The rates of infection with DENV-2 ranged from 21.6% to 90.0% in batches of F_1 females of different strains. When the strains were grouped by geographical region, significant differences in susceptibility were recorded from within certain regions, but not all. Strains obtained from the outskirts of Rio de Janeiro differed in susceptibility, but strains from urban districts or from slums within the city did not (Lourenço-de-Oliveira *et al.*, 2004).

(iii) Genetic differentiation and diversity in populations of *St. aegypti* on two islands (Tahiti and Moorea) in French Polynesia and their susceptibility to infection with DENV-2. Measurements were made of genetic differences at five polymorphic loci in F_1 females, and of susceptibility to infection with a Bangkok strain of DENV-2 on F_1 or F_2 females. On **Tahiti**, the east coast has limited urban development, and *St. aegypti* is found only near isolated habitations, where the larvae inhabit tree holes in rainforest. Here, the populations of *St. aegypti* were genetically more homogeneous and showed low susceptibility to DENV-2. The west coast is highly urbanized, insecticide pressure is high, and *St. aegypti* makes use of artificial containers for larval habitats. In contrast, the populations were genetically heterogeneous, and susceptibility rates ranged from low to high. On **Moorea**, both human density and insecticide pressure are higher on the east coast than on the west coast. The east coast populations of *St. aegypti* were more differentiated genetically than those of the west coast; however, on both coasts *St. aegypti* populations were heterogeneous for susceptibility. On both islands, differences in the genetic structure of the *St. aegypti* populations on the east and west coasts were associated with human population densities and with types of mosquito ecotope. The authors surmised that in the highly urbanized areas on Tahiti, application of insecticides after dengue outbreaks caused recurrent extinction events, leading to differences between the mosquito populations. In less populated zones, where natural larval habitats predominated, the uniform nature of the larval habitat could explain the lack of differentiation between populations (Vazeille-Falcoz *et al.*, 1999; Paupy *et al.*, 2000).

(b) Mosquito hosts: genetics and mechanisms of refractoriness

Susceptibility to oral infection with DENV is a heritable character. An early study involved 13 strains of *St. aegypti* of geographically different origins. Strains that were more susceptible to infection with one of the four DENV serotypes were also more susceptible to the others. As would be expected, the amount of virus required to infect females varied inversely with susceptibility. Crossing susceptible × resistant strains resulted in F_1 progeny with the characteristics of the resistant parent; therefore, susceptibility to infection was recessive and refractoriness was dominant (Gubler *et al.*, 1979). Generally similar results had been obtained with 13 geographic strains of *St. albopicta*, except that crosses between susceptible and refractory strains produced F_1 progeny of intermediate susceptibility (Gubler and Rosen, 1976). In strains of *St. aegypti* from Mexico and the southern USA, the midgut infection rates among females that had imbibed DENV-2 were positively correlated with dose. However, among females with infected midguts, there was no correlation between the amount of virus that had been ingested and the percentage developing disseminated infections (Bennett *et al.*, 2002).

Refractoriness can involve midgut infection and escape barriers. In a West African strain of *St. a. formosa* (from Ibo, Nigeria) refractory to infection with DENV-2, 75% of females had a midgut infection barrier. Of the 25% in which a midgut infection developed, 46% had a midgut escape barrier, and dissemination of virus beyond the

midgut occurred in only 13.5% of females. In a DENV-2 susceptible Puerto Rican strain of *St. a. aegypti*, fewer females (39%) had a midgut infection barrier. A disseminated infection developed in all of the 61% of females in which midgut tissues became infected, therefore none had a midgut escape barrier. Crossing experiments suggested that at least two genes or sets of genes control susceptibility to infection in *St. aegypti*, one controlling the midgut infection barrier, the other the midgut escape barrier (Bosio *et al.*, 1998).

After further crosses between the same susceptible and refractory strains just discussed, quantitative trait loci (QTLs) affecting susceptibility to infection with DENV-2 were mapped in the F_1 progeny, while the F_2 progeny were analysed for midgut infection and escape barriers. QTLs for a midgut infection barrier were detected on chromosomes-2 and -3; they accounted for ~30% of the phenotypic variance in infection, and for 44% and 56%, respectively, of the overall genetic variance. QTLs of minor effect were detected on chromosomes-1 and -3, and some evidence was obtained for a QTL for a midgut escape barrier on chromosome-3 (Bosio *et al.*, 2000). Later, Gomez-Machorro *et al.* (2004) crossed the *Ibo 11* strain of *St. a. formosa* selected for refractoriness to infection with DENV-2 with the *DS3* strain of *St. aegypti* selected for susceptibility. In the F_5 generation of an advanced intercross line, QTLs affecting the midgut infection barrier to DENV-2 were mapped. A new sex-linked QTL (chromosome-1) and a second QTL on chromosome-2 were found to condition midgut susceptibility. Alleles at these QTLs contributed additively in determining susceptibility, and accounted for ~24% of the phenotypic variance.

(c) Mosquito hosts: transmission to primates

The domestic and peridomestic forms of *St. aegypti* are always associated with human settlements, and most of their blood meals are taken on human hosts. In some populations of *St. aegypti*, house-dwelling females take more than one blood meal during a gonotrophic cycle, possibly stimulated by the close presence of hosts. Adult female mosquitoes feed on both nectar and blood, but different populations of *St. aegypti* differ greatly in the extent of nectar feeding. Different behaviour patterns of *St. aegypti* were observed at locations that differed in availability of food sources. The investigators suggested that, where there is a shortage of human and other hosts, female *St. aegypti* use plant sugars as an energy source, but that, where they live in close association with dense human populations, they use blood as an energy source (Volume 2: Sections 36.6, 39.5.5, 39.6.1). Multiple feeding on human hosts is likely to increase virus transmission.

Transmission of DENV-2 was investigated with females of *St. aegypti* that had been orally infected by imbibing an erythrocyte-virus suspension. When infective, these females were either allowed to feed on guinea pigs or allowed to probe but not feed. Their subsequent 'transmission capabilities' were tested *in vitro* by collecting saliva in a serum-containing capillary tube and assaying for DENV-2. Control females that had neither probed nor blood fed since becoming infective had a transmission capability of 66.7%, whereas infective females that had probed 20 times but not blood fed had a transmission rate of 100%. This showed that multiple host contacts do not significantly deplete dengue virus from the salivary glands, and that infective females should be able to transmit to a number of hosts during a single gonotrophic cycle. Infective females that had blood fed and that were tested 48 h later had a transmission rate of 73.7%, compared with the 84.2% of the unfed controls (Putnam and Scott, 1995a). The mean times spent by females probing and then engorging on guinea pigs were not significantly different whether the salivary glands were uninfected or were infected with DENV-2 (Putnam and Scott, 1995b).

To find the amount of dengue virus needed to infect rhesus monkeys, serial dilutions of multi-passaged DENV-2 and DENV-4 were inoculated subcutaneously into groups of monkeys (*Macaca mulatta*), and seroconversions were determined 30 and 60 days later. From titration in *Toxorhynchites* of the serially diluted inoculants, it was found that, to

infect 50% of monkeys, 9.5 MID_{50} (50% mosquito infective dose) of DENV-2, or 22 MID_{50} of DENV-4, was needed (Kraiselburd *et al.*, 1985).

(d) Primate hosts: responses to infection

Infection of primates with a dengue virus quickly elicits an immune response. Infection of humans with a particular serotype results in lifelong immunity to that serotype (homologous immunity) and, for a period of some months, immunity to the other serotypes (heterologous immunity). Types of antibody differ in specificity: neutralizing antibodies are serotype specific; haemagglutination-inhibition (HI) antibodies are usually cross-reactive but do not persist; and complement-fixation (CF) antibodies become increasingly specific with time (Rudnick, 1976; Focks *et al.*, 1995).

In Thailand, where dengue is endemic, 2000 mothers of mean age 26.4 years (range 15–45 years) were tested for antibodies to the dengue viruses: 96.9% were seropositive, most having antibodies against all four serotypes. In 250 randomly selected mother–infant pairs, among which 242 (96.8%) of the mothers were seropositive for antibodies to DENV, all of the seropositive mothers transferred antibodies to their babies. The half-life of the maternally derived antibodies was *c.* 42 days, and all infants lost their antibodies by 12 months of age (Watanaveeradej *et al.*, 2003).

Two or more serotypes may sequentially infect one host. When infection with a second serotype occurs, the antibody response to the sequential, or secondary, infection is markedly different from that elicited by the primary infection. In primary infections, only low levels of IgG are detectable in the febrile or early convalescent phase of infection, whereas the levels of IgM are high. In secondary infections, high levels of IgG are detectable even in the acute phase, whereas IgM levels are low to absent. A secondary antibody response to DENV infection can occur even when the host has previously been infected with a flavivirus that is not a member of the dengue virus group (Innis, 1997). The phenomenon of 'antibody-dependent enhanced infection' in secondary infections is described in Section 44.10.3.b. The pathophysiological effects in humans of infection with dengue viruses are described in that same section.

45.2.6 Sylvatic, rural and urban cycles in Malaysia

Investigations into the ecology of dengue virus in Peninsular Malaysia were initiated in 1962, triggered by an outbreak of DHF/DSS on Penang Island (now Pinang), and continued until 1980. The principal objectives were to test the hypothesis that dengue is a zoonosis and to search for relationships between a postulated jungle (sylvatic) cycle, endemic dengue in rural and urban areas, and urban epidemics. Research was undertaken in a variety of natural habitats (dipterocarp forest, freshwater peat-swamp forest, mangrove-swamp forest and forest fringe); in modified habitats (rubber, oil palm and coconut plantations); and in villages, towns and cities. The forests contained abundant monkey populations but few humans; *St. aegypti* was absent. The findings were described and reviewed by Rudnick (1965, 1976, 1978, 1983, 1986a,b), Rudnick and Chan (1965) and Cheong (1986), from which sources the following account is taken.

(a) Viruses

Several cross-reacting flaviviruses were present, but discriminating tests showed that dengue virus was responsible for most occurrences of flavivirus antibodies in humans residing in jungle, including some at very isolated locations in deep jungle. Dengue virus serotypes 1, 2 and 4 were isolated from sentinel leaf monkeys in the high canopy, where one sentinel leaf monkey also seroconverted for DENV-3. All four DENV serotypes were isolated from humans in jungle.

(b) Vertebrate hosts

Serological tests on >8000 vertebrates indicated that, of the many higher taxa represented, only primates were significantly involved in DENV

transmission cycles; antibodies to other flaviviruses and to alphaviruses were common in domesticated animals, including pigs. Virological and serological investigations on monkeys involved either capture–release–recapture of wild monkeys to obtain series of blood samples, or maintenance of caged sentinel monkeys in the canopy and at ground level to obtain evidence of transmission to the monkeys and to trap mosquitoes that came to them.

Of 585 monkeys taken in selected forest habitats, 68% had anti-flavivirus HI antibodies at a titre of 1:20 or greater, most of which were shown by PRNT to be antibodies to DENV; 8% had only DENV-specific antibody. DENV-specific antibodies were found in recaptured monkeys from mangrove forest, freshwater peat forest and primary dipterocarp forest.

During 1975 in Gunong Besout Forest Reserve, a region of undisturbed hill forest, 27 monkeys, of two species of macaque (*M. fascicularis* and *M. nemestrina*) and three species of leaf monkey (*P. melalophos*, *T. cristatus* and *T. obscurus*), were kept in cages at 23 m elevation in the canopy for an average of 262 days, while at a nearby station 19 monkeys (two species of macaque and one of leaf monkey) were kept in cages at ground level for an average of 423 days. Five of the monkeys kept in the canopy developed dengue infections, and dengue virus was isolated from four of them. None of the monkeys kept at ground level over the same period developed dengue infections. No clinical symptoms were observed in monkeys naturally infected with DENV.

Tests on >2600 human serum samples, including 600 from aboriginal inhabitants, showed a relatively high prevalence of dengue-specific antibody in rural and forest-dwelling people compared with the urban populations.

(c) Mosquito hosts

More than 500,000 biting arthropods, including 300 mosquito species of 20 genera (as then recognized), were collected from a wide variety of habitats. Dengue virus was isolated from *St. aegypti* in urban areas, and from *St. albopicta* in rural areas that appeared to be free from *St. aegypti*. Circumstantial evidence suggested that, among >120 mosquito species captured in the forest, the sylvatic vectors of DENV probably were species of *Downsiomyia*. DENV-4 was isolated from a pool of 21 *Downsiomyia* (probably *D. pseudonivea/D. subnivea*) captured in a monkey-baited trap in the high canopy of Gunong Besout Forest Reserve. The sentinel monkey in the trap had seroconverted from DENV-4 some 3 months earlier. No isolations were made from other arthropod species.

On dates when sentinel monkeys became infected, comparison of the mosquito species captured in a baited trap in the canopy with the species captured in baited traps on the ground supported the view that species of *Downsiomyia* were the most likely vectors in deep primary forest, where they were the most common diurnal mosquitoes in the high canopy. In 1974 and 1975, species of *Downsiomyia* formed 11.0% and 13.3% of all mosquitoes collected in the forest. In 1976, in collections that compared responsiveness to human bait at ground level and in the high canopy, 2.2% of *Downsiomyia* were caught at ground level and 97.8% on high-canopy platforms (n = 1920). The females had a feeding preference for primates (including humans), and engorged specimens were caught in the high-canopy trap whenever the sentinel monkeys became infected. Among the 11 species of *Downsiomyia* then recognized in Peninsular Malaysia, *Do. pseudonivea* and *Do. subnivea* were the most widespread, and were the most frequently collected in primary hill dipterocarp, lowland dipterocarp and freshwater peat-swamp forests. In those habitats, the two species always occurred together, almost always in the high canopy. In contrast, in 'man-disturbed' hill dipterocarp forest, *Do. novonivea* was the commonest canopy species of the genus, while in mangrove swamp forest *Do. litorea* and *Do. leonis* were the dominant canopy species. No *Downsiomyia* were detected in oil palm or coconut/cocoa estates.

Stegomyia albopicta was an endemic species which occurred in small numbers in the deep forest, where it was active principally at ground level. It occurred commonly at the forest fringe, in

agricultural areas, villages and urban areas, and was thought to provide an important link in the transmission of DENV between jungle, rural and urban communities. The females were diurnal and fed primarily outdoors. The larvae developed in natural and human-made containers. At the time of these studies, the invasion of urban habitats in South-east Asia by *St. aegypti* was advanced, and *St. aegypti* had largely replaced *St. albopicta* in urban habitats (Rudnick, 1983; 1986a,b; Rudnick and Chan, 1965).

(d) Transmission cycles

Dengue viruses were isolated from sentinel leaf monkeys kept in a canopy trap 23 m above the forest floor; macaques kept in a ground trap during the same period were not infected. Throughout the period of study there was no evidence of dengue infection in any sentinel monkeys kept at ground level. Wild macaques spent considerable time on the ground but also ascended into the canopy. Wild leaf monkeys spent >90% of their time in the canopy but occasionally descended to the ground. It became apparent that all four dengue serotypes circulated between monkeys and mosquitoes in the high canopy of primary undisturbed jungle. There was no transmission to humans; *St. albopicta*, when present, was active only at ground level, and *St. aegypti* was absent. The evidence suggested that species of *Downsiomyia* were the most probable vectors in this silent cycle.

Stegomyia albopicta and *St. aegypti* transmitted dengue viruses between humans. *Stegomyia albopicta* was widespread, being found in jungle, rural areas, suburban areas and cities; its densities were highest in rural and suburban areas. It lived around human dwellings, and was found most commonly outdoors where it fed on humans. *Stegomyia aegypti* was synanthropic; it was absent from forest and some rural areas, but was abundant in villages, in which the households had many containers for collecting rainwater. The larvae developed in water-filled containers in and around human dwellings, and the adult females were mostly found inside houses, where they fed avidly on humans. *St. aegypti* occurred in greatest numbers in crowded urban centres, and decreased away from centres of human population (Gill, 1976; Rudnick, 1976).

Dengue has long been endemic in Malaysia, small numbers of cases being reported throughout the year. Most Malaysians have developed antibodies to dengue virus by the time they reach adulthood. Dengue epidemics, which have been recorded in Malaysia since the beginning of the 20th century, occurred almost exclusively in urban situations, where the clinical cases were of either classic dengue fever or DHF/DSS. During the second half of the 20th century, urbanization increased markedly; the expanding urban centres were continually supplied with large numbers of non-immune humans, while urbanization favoured the growth of *St. aegypti* populations. In the rural areas, in contrast, the ecological conditions favoured *St. albopicta*, human population numbers were stable, and the numbers of immunes changed only slowly.

Studies undertaken in agricultural and rural areas, from which *St. aegypti* was absent, revealed an association between *St. albopicta* and a mild, endemic dengue fever that usually evaded clinical diagnosis. Prevalence was highest in people associated with rubber plantations adjacent to forested areas. In urban areas where both *St. aegypti* and *St. albopicta* occurred, the clinical manifestations of dengue infection ranged from mild to fatal. In outbreaks of classic dengue fever, the distribution of patients correlated positively with the distribution of *St. albopicta*. In contrast, in each DHF/DSS epidemic, the distribution of patients correlated positively with the distribution of *St. aegypti* (Rudnick *et al.*, 1965, 1976; Rudnick, 1976, 1983, 1986a; Gill, 1976).

(e) Summary

In Peninsular Malaysia during the second half of the 20th century, dengue virus circulated in three linked transmission cycles: (i) a silent, enzootic, sylvatic cycle involving monkeys and species of *Downsiomyia* in the forest canopy, and with some transmission to members of aboriginal groups on

the ground; (ii) an endemic rural cycle, involving humans and *St. albopicta*, and causing a febrile illness of short duration which usually evaded diagnosis; and (iii) an urban cycle which usually was endemic but had occasional epidemics, involving *St. albopicta* and/or *St. aegypti* as vectors, and with clinical manifestations ranging from mild febrile episodes to classical DF to DHF/DSS. The distribution of DHF/DSS patients correlated with the distribution of *St. aegypti*. Initially at least, dengue was a zoonosis: probably, *St. albopicta* transmitted DENV from monkeys to humans at the forest edge, and from there to humans in villages, towns and cities.

Silent cycles of DENV in the forest canopy might once have been characteristic of all primary forests in South-east Asia. Sera obtained in 1963–1966 and 1978 from macaques imported from five South-east Asian countries, and tested by measurement of PRNT, showed that 15% to 87% had been infected with one or more dengue viruses (Yuwono *et al.*, 1984). Discussing an epizootic in a wild population of toque macaques in Sri Lanka (South Asia) in 1986–1987, de Silva *et al.* (1999) considered that such outbreaks might have implications for public health. However, the steady reduction of tropical forest in South-east Asia, and consequently of the monkey populations, has diminished populations of these amplifying host of dengue virus, so the sylvatic cycle may become largely relict. Urbanization without adequate controls provided conditions suitable for the establishment of *St. aegypti* populations and, through the continual influx of immunes, for the maintenance of dengue virus.

45.2.7 Sylvatic cycles in West Africa

Molecular-genetic data suggest that sylvatic strains of DENV-2 reached Africa hundreds of years ago (Section 45.2.1), but there is scant information on the history of dengue as a human disease in that continent. In 1927, at a time when dengue was not endemic in South Africa, a massive dengue epidemic occurred in Durban (Edington, 1927). Describing dengue outbreaks in Ethiopia, Somalia and Madagascar during the years 1941–1945, McCarthy and Bagster Wilson (1948) stated that dengue had been recognized for many years on the East African coast. However, not until 1964–1968, in Nigeria, were DENV-1 and DENV-2 identified in isolates from human cases (Carey *et al.*, 1971). The isolation of DENV-2 in Senegal in 1970 triggered intensive investigations into DENV transmission in that country, particularly in the department of Kédougou, an area of savannah and forest galleries surrounding the town of Kédougou (12° 11' W, 12° 33' N). Being a part of the Sudano–Guinean bioclimatic zone, this is in a region of high rainfall (1200–1300 mm p.a.). The rainy season usually lasts from May/June to October/November, with maximum rainfall in August or September. The population of the area is essentially rural (Diallo *et al.*, 2003).

The findings from Senegal, which probably can be taken as representative of West Africa, revealed sylvatic cycles of DENV-2 involving monkeys as the primate hosts and aedine mosquitoes as putative vectors. Sylvatic outbreaks were periodic, appearing after silent intervals lasting 5–8 years, and were recorded in 1974–1975, 1980–1982, 1989–1990 and 1998–2000. Human infections were rarely reported, and any clinical effects were mild (Diallo *et al.*, 2003).

An early retrospective investigation involved serological study of samples obtained from simian and human populations in the Kédougou area, when 1095 simian sera collected between 1974 and 1984 were analysed, as were 1783 sera from children aged <11 years collected after each rainy season from 1976 to 1985. It transpired that epizootics of DENV-2 had occurred in simian populations in the Kédougou area in 1974–1975 and 1981–1982. Positive sera were obtained from *Erythrocebus patas*, *Chlorocebus aethiops* and *Papio hamadryas*. No antibodies to dengue virus were detected in sera from children until the 1981–1982 epizootic in monkeys, when about 11% of the sera revealed a probable infection by DENV-2. No clinical signs of dengue fever had been reported (Saluzzo *et al.*, 1986).

In investigations conducted in the Kédougou area in 1999 and 2000, mosquitoes captured in human-biting catches during June, October and November 1999 were screened for DENV-2. No isolates were obtained in June (n = 8157). Sixty-four isolates were obtained from females captured during October and November (n = 16,506), distributed among species as follows: *Diceromyia furcifer*, 35; *Di. taylori*, 11; *St. luteocephala*, 16; *St. aegypti*, 2; the mean infection rates did not differ significantly (p = 0.34). Although the vector populations were high at the beginning of the rainy season, appearance of the virus and the period of its amplification occurred only at the end of the rainy season. Of the 64 isolates, 58 were from mosquitoes caught in the forest gallery, and 6 from mosquitoes caught in villages. In the forest gallery, *Di. furcifer*, *Di. taylori* and *St. luteocephala* were very aggressive to humans. *Stegomyia aegypti* showed only weak human-biting activity, consistent with its presence in West Africa in its sylvatic form, *St. aegypti formosa*. In the Kédougou area during October and November 1999, *Di. furcifer* was estimated to be responsible for at least two infectious bites per person per week, and in Bandafassi village for at least two infectious bites per person each evening (Diallo *et al.*, 2003).

During 3 months of the 2000 rainy season (August, October, November), extensive collecting in the same locations as in 1999 yielded only nine isolates of DENV-2 from female mosquitoes: *Di. furcifer*, 1; *Di. taylori*, 2; *St. luteocephala*, 2; *St. aegypti*, 2; *Fredwardsius vittatus*, 2. Blood samples from 17 vervet monkeys (*Chorocebus aethiops*; cited as *C. sabaeus*) captured in a forest gallery in early February 2000 were tested by ELISA for anti-DENV-2 IgM and IgG. All were negative for the IgM, but ten were positive for the IgG. Assuming no cross-reaction with other flaviviruses, those ten monkeys had been hosts of DENV-2, but their infections had not been recent. DENV-2 was isolated from *Di. furcifer* in all villages where human-biting catches were undertaken, but no clinical cases of DENV-2 were recorded in the area. Diallo *et al.* (2003) concluded that DENV-2 is transmitted in a sylvatic cycle in Senegal, in which *Di. furcifer*, *Di. taylori* and *St. luteocephala* are putative vectors. DENV-2 is essentially confined to the forest, but *Di. furcifer* carries it from the forest galleries to nearby villages. In all villages where mosquito catches were undertaken, DENV-2 was isolated from *Di. furcifer* caught in peridomestic habitats, but no human clinical cases were recorded from the region. Where transmission to humans occurs, the rate is low and any infections result in non-symptomatic cases.

The period of virus appearance and maximum amplification coincided with the end of the rainy season, possibly owing to the greater longevity of vectors at that time. Little is known of the rate of turnover of susceptible primate hosts, and the serological data obtained from monkeys were insufficient to establish their role as amplifying hosts. Isolations from adult male mosquitoes showed that DENV-2 is transmitted from generation to generation vertically as well as horizontally. Such isolates were obtained from Furcifer Group males in Côte d'Ivoire (Cordellier *et al.*, 1983; Roche *et al.*, 1983); one isolate was obtained from *Di. taylori* in Kédougou, Senegal (Saluzzo *et al.*, 1986); and two isolates were obtained from male *Di. furcifer* in Kédougou (Diallo *et al.*, 2003). Culturing tree-hole samples collected in the Kédougou area during the 2000 dry season led to the emergence of 2423 adult aedine mosquitoes, of which 94.1% were species of *Stegomyia*. None of the emerged adults was infected with DENV-2. The importance of vertical transmission for the survival of DENV-2 through the dry season has not been evaluated.

To test the susceptibility of putative vector species to infection with sylvatic and epidemic strains of DENV-2, mosquitoes were fed blood containing low-passage DENV-2 of three West African strains – two sylvatic and one epidemic. The mosquitoes were F_1 offspring of wild-caught females from sylvatic populations of *Di. furcifer*, *Fr. vittatus*, *St. luteocephala* and *St. aegypti*, and from a peridomestic population of *St. aegypti*. *Di. furcifer* was moderately or highly susceptible to both the sylvatic and epidemic strains of DENV-2, whereas *St. luteocephala* was highly susceptible to both strains (Table 45.6). Tested only against the two sylvatic strains, *Fr. vittatus* showed

Table 45.6 Susceptibility of putative vector species, collected from sylvan or peridomestic habitats in Senegal, to infection with sylvatic or epidemic strains of Dengue virus 2 of West African origin. (From Diallo *et al.*, 2005b.) In some cases, the authors recorded the results of duplicate tests with particular virus strains against individual mosquito species.

Species	*Virus strain*	*Virus titre* ($TCID_{50}$ ml^{-1})	n	*Infection rate* (%)
Diceromyia furcifer (sylvan)	1349 (epidemic)	6.5	18	83
	1349 (epidemic)	9.2	30	77
	PM33974 (sylvatic)	8.0	30	97
	DakAr2022 (sylvatic)	7.0	23	26
	DakAr2022 (sylvatic)	7.9	37	35
Stegomyia luteocephala (sylvan)	1349 (epidemic)	8.2	18	89
	PM33974 (sylvatic)	5.5	21	67
	PM33974 (sylvatic)	8.0	12	67
	DakAr2022 (sylvatic)	9.2	19	58
	DakAr2022 (sylvatic)	8.2	19	79
Fredwardsius vittatus (sylvan)	PM33974 (sylvatic)	8.8	36	6
	DakAr2022 (sylvatic)	6.5	54	19
Stegomyia aegypti (sylvan)	1349 (epidemic)	8.0	20	0
	1349 (epidemic)	8.8	24	25
	PM33974 (sylvatic)	8.7	17	0
	PM33974 (sylvatic)	8.2	17	0
	DakAr2022 (sylvatic)	8.2	27	15
St. aegypti (peridomestic)	1349 (epidemic)	8.8	38	10
	DakAr2022 (sylvatic)	7.2	32	3

Virus strains: 1349, ex human, Burkina Faso, epidemic; PM33974, ex *St. africana*, Guinea, sylvatic; DakAr2022, ex *St. africana*, Burkina Faso, sylvatic.
The mosquitoes were F_1 offspring of wild-caught parents from sylvatic populations in Kédougou forest galleries (SE Senegal), or from a peridomestic population in the town of Koung Koung (central Senegal). They were fed blood containing 5.5–9.5 $TCID_{50}$ (50% tissue-culture-infective dose) ml^{-1} of low-passage DENV-2, and infection rates were recorded.

low susceptibility to both. The sylvatic *St. aegypti* were non-susceptible or weakly susceptible to both epidemic and sylvatic strains of DENV-2. Exposed to one epidemic and one sylvatic strain of DENV-2, the peridomestic *St. aegypti* were only weakly susceptible to both (Diallo *et al.*, 2005b).

Epizootics of DENV-2 that have been reported from other West African countries, including Burkina Faso and Côte d'Ivoire, resembled those in Senegal in a number of ways: they involved cycles of sylvatic transmission, with monkeys as the primate hosts and aedine mosquitoes as vectors; and few or no human cases were described (Cordellier *et al.*, 1983; Hervy *et al.*, 1984; Robert *et al.*, 1993). Only one human epidemic, which occurred in Burkina Faso in 1982, has been reported from West Africa; nucleotide sequencing showed the causal agent to be closely related to a strain of DENV-2 from the Seychelles, and presumably imported by a traveller (Rico-Hesse, 1990).

From the evidence available, dengue in Africa is characterized by predominantly sylvatic trans-

mission cycles and by the absence of severe clinical effects in infected humans. Possible explanations that have been suggested variously concern the virus, the human hosts and the mosquito vectors. Possibly the African strains of DENV-2 have a low capacity for infecting humans or a low pathogenicity in humans; possibly, people of African descent are less disposed to severe dengue than peoples of South-east Asia; possibly, the African mosquitoes have lower vectorial competence (François Rodhain, personal communication; Diallo *et al.*, 2005b). The answers are still awaited.

45.2.8 Urban cycles

(a) Characteristics

Although a zoonosis, dengue fever is often considered a predominantly human disease, occurring in towns and cities of the tropics and subtropics where the viruses are maintained in human/*St. aegypti* transmission cycles. In most tropical or subtropical towns and cities in which dengue viruses are present, the domestic form *St. aegypti aegypti* is the sole vector, reflecting the wide distribution of this subspecies and its synanthropic characteristics.

Service (1989) described the growth of urban centres and the attendant risks of arthropod-borne infections. Historically, where human populations grew the villages also grew, and some expanded into towns and cities. Often, human wastes, which previously had been used or scattered, began to accumulate, and before long pollution and slum conditions arose. Polluted waters on the ground provided larval habitats for *Culex quinquefasciatus* and, commonly, bodies of clean water in storage vessels or discarded containers provided larval habitats for *St. aegypti*. Humans served as blood-meal sources for adults of both species. The risk of epidemics of mosquito-borne infections was high when an abundance of larval habitats was combined with dense populations of susceptible hosts. The largest urban populations at risk are found in the cities of the wet tropics.

The cities of South-east Asia have long harboured dengue viruses, and in recent decades these viruses have dispersed widely across the world, reaching East Africa, the Caribbean and other parts of the Americas. In the New World tropics, dengue viruses are perpetuated only in urban cycles. In some large conurbations, dengue fever is endemic, with seasonal transmission and often more than one serotype circulating. Usually, major epidemics occur in cities where most of the population is non-immune. Human immune responses prevent reinfection with the same serotype; after epidemics, any persistence of dengue viruses requires a high human birth rate and an abundant vector population. Even so, epidemics soon recur in some tropical cities. Outbreaks of dengue fever occur also in rural environments (Kuno, 1997, review), but to the extent that they involve transmission between humans and *St. a. aegypti* they can be regarded as urban cycles. In locations with smaller human populations, characteristically, after a period of intense transmission, recognizable cases of dengue disappear. In areas with much larger populations dengue often becomes endemic.

(b) Dispersion of vector and virus within cities

Reports of the clustering of dengue cases within households over short periods of time stimulated Getis *et al.* (2003) to characterize the spatial distribution of *St. aegypti* in two neighbourhoods in Iquitos, Peru, a city of about 345,000 people isolated in the Amazon forest. In each neighbourhood, house-by-house counts were made of the numbers of *St. aegypti* larvae, pupae and adults. Global *K*-functions (a global spatial statistic) were used to identify clustering in each neighbourhood, and the local statistic G_1^* was used to score the magnitude of variables in each household. The adult mosquitoes were strongly clustered within distances of 10 m. Such a distance might be within a single house, or it might extend to two or even three houses where the house widths were narrow. In addition, the adults clustered weakly to a distance of 30 m beyond the household. Beyond 30 m, any further clustering of adult mosquitoes corresponded to the clustering of houses. Pupae clustered strongly within houses, but did not cluster beyond the

household; more widely, they were dispersed rather evenly throughout the neighbourhood. The authors concluded that, over short periods of time, the frequent blood-feeding activities of *St. aegypti* and their restricted flight range were underlying factors in the clustered patterns of human dengue infections.

The distances flown by *St. aegypti* are affected by characteristics of the habitat – notably distances to hosts or to oviposition sites. Substantial differences in flight range have been reported from within open desert, villages and cities (Volume 2, Section 33.9.2.a). Rubidium has proved useful as a marker for measuring flight distances in cities. Adult females are fed blood laced with rubidium chloride, and released; during oogenesis rubidium accumulates in the oocytes, and later provides a marker for eggs retrieved in ovitraps (Reiter *et al.*, 1995). In a study in San Juan, Puerto Rico, nulliparous females reared from locally collected eggs were fed rubidium chloride and 11.5 h later 90 females were released at the centre of an area containing 550 ovitraps. The study area was roughly circular, with an average radius of 420 m. The ovitraps were replaced daily. On the day of first collection, marked eggs were found at 68 sites, and at a mean distance of 181 m (range 9–432 m) from the release point. In three subsequent daily collections, the mean distances from the release point were: 237 m (33–417 m); 221 m (99–417 m); and 279 m (39–441 m). This revealed rapid dispersion over the entire 55 ha study area (there was no obvious effect of wind). The investigators concluded that the dispersion of *St. aegypti* may be driven by the availability of oviposition sites (Reiter *et al.*, 1995; Reiter, 1996). In Singapore, similar use of RbCl as a marker showed that *St. aegypti* dispersed quickly through an area of 320 m radius in quest of oviposition sites. Release from the 12th floor of a 21-floor apartment blood, with ovitraps on each floor, showed rapid dispersion to the top and bottom of the block (Liew and Curtis, 2004).

Females of *St. aegypti* tend to lay a few eggs at each of a number of sites, so local populations can be genetically heterogeneous (Volume 2; Section 40.11.1.e). The distance over which females of *St. aegypti* disperse can vary with the habitat, for example differing between a village and a town. Even within a city, flight distances can vary if its neighbourhoods are sufficiently diverse. For these reasons, genetically distinct subpopulations of *St. aegypti* can exist in different parts of a city. In Cali, Colombia, where dengue was hyperendemic, populations of *St. aegypti* from different areas of the city were genetically heterogeneous, and their vectorial competence for two laboratory strains of DENV-2 ranged from 19% to 60% (Ocampo and Wesson, 2004).

During the acute phase of dengue fever, high-titre viraemia persists for 1 or 2 days. In any *St. aegypti*-infested location where people congregate, transmission from infective to non-infected humans can occur, and the tendency of this mosquito to take supplementary blood meals during a single gonotrophic cycle may increase transmission rates. In households that contain several people, often a number are infected. Because *St. aegypti* does not disperse over great distances, long-distance dissemination of dengue viruses is more likely to be due to travelling, infective humans. As a quite different possibility, an influx of non-immune individuals into a dengue-endemic area can result in an outbreak (Rodhain and Rosen, 1997; Deubel and Murgue, 2001).

(c) Seasonality

In many places, dengue epidemics typically occur during the wet or rainy season, a time when mosquito populations increase. For example, during an epidemic of DHF on Koh Samui Island in the Gulf of Thailand, the incidence of DENV-2 infections in both humans and mosquitoes was highest during the period of greatest precipitation, and the densities of *St. aegypti* and *St. albopicta* were said to be 'directly related to the amount of rainfall' (Gould *et al.*, 1970). From such correlations, some authors have inferred that dengue epidemics result from an increase in number of vectors. However, in some locations, dengue outbreaks may occur at the end of or outside the rainy season (Kuno, 1997); and, further, when dengue epidemics occur during

a rainy season there is not always an associated increase in vector populations, as the following investigations showed.

In Bangkok and its surrounding areas there are three main seasons, the timing of which varies slightly from year to year: a cool-dry season (November/December to February), a hot-dry season (March to April/May), and a rainy season (May/June to November) (Figure 45.11). DHF incidence begins to increase during the latter half of the hot-dry season and reaches a maximum during the rainy season, before subsiding markedly during the cool-dry season (Watts *et al.*, 1987). Investigations into the life budgets and population dynamics of *St. aegypti* in Bangkok, conducted over a number of years, provided a basis for assessing whether seasonal changes in its population densities were causally associated with the incidence of DHF. It was found that: (i) there was some reduction in the larval population during the cool-dry and hot-dry seasons; (ii) fluctuation in the number of adult mosquitoes was determined by the mortality of the immature stages; (iii) changes in adult population size were not correlated in any simple way with temperature or rainfall and, in particular, there was no striking increase in adult population size in the wet season. It seemed unlikely that outbreaks of DHF could be explained by increases in *St. aegypti* densities during the wet season (Sheppard *et al.*, 1969; Tonn *et al.*, 1969, 1970; Southwood *et al.*, 1972). From the data produced by these investigators, Dye (1984) developed analytical and multi-age-class simulation models which predicted that fluctuations in adult population size were due to density-dependent larval mortality coupled with fluctuations in adult survivorship.

Other investigators offered a different explanation for dengue seasonality in Bangkok. A study of blood feeding and ovary development in *St. aegypti* at different times of year revealed a delay of 1–2 days in first feeding in the cool-dry season, a shortening of the gonotrophic cycle in the hot-dry and rainy seasons, and an apparent shorter adult survivorship in the cool-dry season. Pant and Yasuno (1973) suggested that the incidence of DHF might be lowest during the cool-dry season because of a reduction in biting rates owing to a delay in first feeding and a lengthening of the gonotrophic cycle. The problem was re-examined by Watts *et al.* (1987) in a laboratory study of the effects of temperature on the rate of infection and speed of dissemination of DENV-2 in *St. aegypti* and of its transmission to monkeys (Section 44.8.4). They made two pertinent observations: (i) DENV-2 was transmitted to monkeys only by females kept at ≥30°C; (ii) the extrinsic incubation period declined from 12 days at 30°C to 7 days at 32°C and 35°C. Noting that the mean temperatures in Bangkok were 28–30°C during the period of high DHF incidence, and 25–27°C when its incidence was low (Figure 45.11), they tentatively concluded that during the cool-dry season the speed of multiplication and dissemination of DENV-2 in *St. aegypti* might be inadequate for effective transmission.

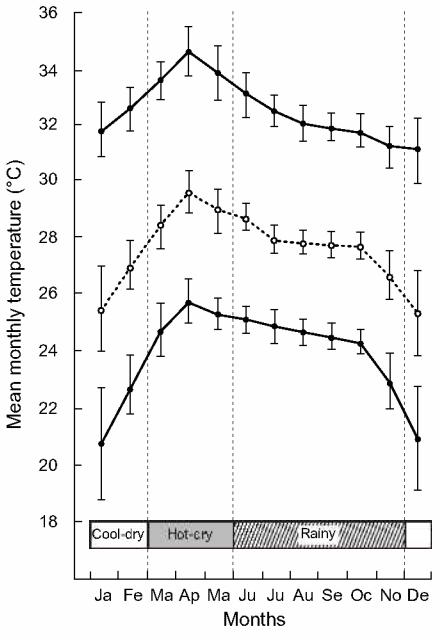

Figure 45.11 Monthly maximum (top curve), mean (middle curve) and minimum (bottom curve) temperatures in Bangkok, 1958–1978 (means and ranges). (After Watts *et al.*, 1987.) Superimposed are the durations of the cool-dry, hot-dry and rainy seasons according to those authors. The incidence of dengue haemorrhagic fever (not shown) begins to increase during the latter half of the hot-dry season, peaks during the rainy season, and subsides markedly during the cool-dry season.

45.3 TRANSMISSION OF YELLOW FEVER VIRUS

45.3.1 Introduction

(a) Historical outline

Historical records from the 17th, 18th and 19th centuries show yellow fever (YF) to have been one of the most important infectious diseases of humans in West Africa and the Americas. It has proved difficult, from the early records, to identify with certainty the continent of origin of yellow fever virus (YFV), but it is now generally considered that it originated in Africa and was carried in ships to the New World, together with the vector *Stegomyia aegypti* (Section 45.2.2.b). Estimates of the numbers of outbreaks of yellow fever in earlier centuries are suspect at best. For the period 1814 to 1900, Scott (1939) found records of only 23 serious outbreaks of yellow fever in West Africa but records of 116 in the New World, mostly in ports on the eastern seaboard. A 'partial listing' of outbreaks of yellow fever in Africa during the period 1940–1986 included 21 epidemics, the most extensive of which, in Ethiopia in 1960–1962, involved an estimated 100,000 clinical cases and 30,000 deaths (Monath, 1989).

Conditions that could permit the identification of the vector of the infectious agent of yellow fever came together when Carlos Finlay, a young physician interested in how outbreaks of yellow fever could be explained, returned to Cuba, where yellow fever was both endemic and epidemic. There, he came to the conclusion that the agents of transmission must be mosquitoes, and that the species concerned was *Culex mosquito* Robineaux-Desvoidy. In 1881, Finlay published a detailed description of the stylets, feeding mechanism and discharge of saliva by female *Cx. mosquito*. He postulated that, when a mosquito feeds on an infected individual, infectious particles might become trapped on setae of the labrum and transferred to a healthy individual when that mosquito next fed. Finlay knew that *Cx. mosquito* fed at intervals of 2 or more days but, unfortunately, he was unaware of the extrinsic incubation period of the infectious agent, which lasts for over 10 days. Consequently, his attempts to achieve transmission experimentally by allowing a mosquito to blood feed on an infected individual and shortly afterwards on a healthy individual failed (Finlay, 1902).

Two major investigations into the transmission of the causative agent of yellow fever were set up in the New World. One, set up in 1900, was the 'United States Army Commission on the Island of Cuba', staffed in 1901 by Jesse Lazear, Aristede Agramontee and James Carroll (who died of yellow fever), and led by Walter Reed. The other was a French Mission in Brazil. After the belief that the infective agent of yellow fever was a bacterium named *Bacillus icteroides* had been disproved, interest turned to Finlay's mosquito theory of transmission, with the American team using mosquito eggs received from Finlay's colony but naming the species *Culex fasciatus* (Spielman and D'Antonio, 2001; Monath, 2010).

During 1898, the epidemiologist Henry Carter, working on yellow fever transmission in Mississippi, found that, after mosquitoes have fed on a yellow fever subject, a period of 10 to 17 days must pass before they have become infective and can infect a healthy person by bite. He referred to that period as 'extrinsic incubation'. According to Richter (1967), in a letter dated 26 July 1900, Carter informed Jesse Lazear of this finding, which he was to publish later that year (Carter, 1900). With that knowledge, the members of the US Commission redesigned their experiments, and achieved transmission of the infectious agent of yellow fever by mosquito bite (Reed *et al.*, 1901).

The work of the US Army Commission and the French Mission led to important advances in knowledge of yellow fever. In Cuba, it was found

that yellow fever could be caused by inoculation with serum filtrate that had passed through a Berkefeld filter that retained bacteria (Reed, 1902). From Brazil, it was reported that the blood of a yellow fever patient contained a virus that was inactivated by warming to 55°C. Inoculation of blood collected on the third day of infection caused yellow fever in the recipient, while inoculation with heat-inactivated serum provided some immunity against injection of low doses of virus (Marchoux *et al.*, 1903). Much later, isolation of YFV by Stokes *et al.* (1928) led to the development of vaccines and to their use, notably in mass immunization with a French neurotropic vaccine in francophone West Africa.

During the period 1985–1999 a total of 25,846 clinical cases of yellow fever worldwide, with high fatality rates and mostly occurring in Africa, were reported to WHO (Monath *et al.*, 1981). However, the accuracy of even official figures such as those has been questioned. Digoutte *et al.* (1995) noted that most published records of fatalities were not supported by serological data, and recommended that they be treated with caution. To adjust for underreporting of numbers of clinical cases and of deaths in YF epidemics, a minimum correction of one order of magnitude or a maximum correction of two may be applied (WHO, 1990). Yellow fever remains a serious problem in West Africa, and to a lesser extent in some other tropical regions.

Yellow fever virus (YFV) can be maintained in two distinct transmission cycles, sylvatic and urban, giving rise to two forms of the disease – sylvatic yellow fever and urban yellow fever. (i) In tropical forests or forested areas, YFV circulates in **sylvatic cycles** between monkey populations and canopy-dwelling mosquitoes. In the main enzootic areas, the tropical forests are large enough for the virus to move in epizootic waves, which carry the virus from one susceptible monkey population to another. The monkeys can be regarded as amplifying hosts. Where humans enter forests to work, or human populations live within or near forests, humans may provide additional mammalian hosts. In East Africa this form of human disease is sometimes called 'rural yellow fever'; in West Africa it has been called 'intermediate yellow fever', and in South America 'jungle yellow fever'. (ii) In some urban centres, and in some areas of savannah where humans greatly outnumber monkeys, YFV circulates in an **urban cycle** in which humans are the vertebrate host and, most frequently, the synanthropic mosquito *St. aegypti* is the vector. Where the population of human susceptibles or the rate of immigration of susceptible humans into urban centres is high enough, humans are the amplifying host (Figures 45.15, 45.16, 45.17).

The greater part of the description of YFV transmission in this chapter is devoted to sylvatic cycles, reflecting the ecological complexity of those cycles and the ways in which they vary between different geographical regions. Only a relatively small part concerns the simpler urban cycle, although it has been responsible for very many human deaths. The pathology of YFV infections in primates is described in Section 44.10.3.a.

(b) Geographical distribution of yellow fever

Yellow fever infections of humans and certain mammals have occurred, broadly speaking, throughout the tropical regions of Africa, the tropical rainforest and grasslands in parts of Central America, and the Amazon and Orinoco river basins of South America. Why yellow fever does not occur in geographical regions with warm climates east of 60° E, including South Asia, South-east Asia and the Pacific region, has been considered problematic, although the question was addressed over half a century ago. Noting that dengue fever had been most heavily reported in places where yellow fever was unknown, May (1952) prepared maps that plotted the world distributions of yellow fever and dengue fever during the period 1930 to 1951 'as described by WHO in 1952'.

In Africa, a broad band of endemic yellow fever extended across the continent between the latitudes of 10° S and 15° N. Thus, the southern boundary extended between mid-Gabon and mid-Kenya, while the northern extremity extended between northern Senegal and northern Eritrea. In South America, the northern boundary of endemic yellow fever was similar to that in Africa,

at about 15° N, but the southern boundary extended slightly beyond 20° S, the whole extending from Panama to Bolivia. The area of endemic YF approached but did not reach the west coast of South America. Small numbers of cases of YF occurred annually in Ecuador, Mexico and the southern USA. The pattern of outbreaks of yellow fever in the New World reported by May (1952) reflected the victory won over the urban cycle by the eradication of *St. aegypti* from Brazil between 1934 and 1940, and from a number of other New World countries in a campaign that started in 1947 (Section 45.2.2.d).

The area of epidemic dengue fever was almost entirely different. It overlapped the area of endemic YF along tributaries of the Nile in southern Sudan, but only there. In North Africa and the southern Palaearctic, it extended along the Nile and into Palestine, Turkey and southern Greece. Much further east it was very extensive, extending from South Asia to South-east Asia, southern China, southern Japan, islands of the Pacific and the east coast of Australia.

May's (1952) map showed isolated areas of occasional dengue fever epidemics within the areas of yellow fever endemicity, but most were away from those areas. Reports examined by Frederiksen (1955) of outbreaks in Havana (Cuba), Martinique, and Charleston, New Orleans, Savannah, Vicksburg and Houston (USA) indicated that only a few of many epidemics of dengue and yellow fever occurred at the same place and during the same year. He noted reports of a similar situation in Senegal in 1878, and concluded that there appeared to be a tendency towards mutual exclusiveness between the two diseases.

(c) Distribution of vectors

The distribution of yellow fever by the mid-20th century, as considered by May (1952), reflected the human-assisted distribution from Africa of *St. aegypti*, a synanthropic and highly efficient urban vector, and the presence in other regions of mosquito species able to transmit YFV. In South and Central America, indigenous species of *Haemagogus* and *Sabethes* were available to serve as sylvatic vectors of YFV between wild primate hosts (Section 45.3.6.d), while *St. aegypti* served as an urban vector of YFV, principally in coastal areas, and in North America too.

Stegomyia albopicta, a competent vector of YFV and DENV, was widely distributed through South Asia, South-east Asia, Japan and some Pacific islands. At that time, it had hardly spread west from India, but had been recorded from Madagascar and nearby smaller islands.

The total absence of yellow fever from India and countries further east, where *St. aegypti* was widely distributed, could not be explained by the absence of an effective urban vector. However, the maps did show the widespread occurrence of epidemic dengue fever in those areas.

(d) Interference between YFV and DENV

Experiments to study possible interference between DENV and YFV were undertaken by inoculating the viruses into rhesus monkeys. Inoculation of the highly virulent Asibi strain of YFV alone produced a clinically severe, fatal disease. When DENV had been injected into seven monkeys 2 or 3 days before the YFV, six of the seven survived, whereas all of nine control monkeys injected with only YFV died. Because *St. aegypti* is a natural vector of both DENV and YFV, and because mosquitoes once infected remain infected for the remainder of their lives, the possibility of interference between the two viruses in mosquitoes was examined. After batches of uninfected (control) and dengue-infected *St. aegypti* had fed on suspensions containing varying amounts of YFV, the extent of replication of the virus in the mosquitoes was determined. When those mosquitoes later fed on monkeys, control mosquitoes that had ingested 10^5 LD_{50} or less of YFV transmitted yellow fever, whereas the previously dengue-infected mosquitoes did not. These results strongly suggested interference with the replication of YFV in dengue-infected mosquitoes. Sabin (1952) surmised that, in an area in which

dengue fever is endemic, a sufficient proportion of the vector population might be sufficiently refractory to the replication of YFV to prevent its establishment.

(e) Genetic analysis of YFV

Yellow fever virus is the type species of *Flavivirus* (family *Flaviviridae*). It is in one of the clusters of flaviviruses that are associated with aedine mosquitoes and primates as hosts, and that cause viral haemorrhagic fever in seriously affected humans (Section 44.1.2.b). The prototype strain of YFV was isolated in 1927 from the blood of a young Ghanaian, and was given his name – Asibi. After some 45 serial monkey passages, this strain was fully sequenced by Hahn *et al.* (1987).

Nucleotide sequencing of structural genes from many strains of YFV revealed considerable genetic variation between strains, but showed that groups of strains that had generally similar sequences came from particular geographical regions (Deubel *et al.*, 1986; Lepiniec *et al.*, 1994; Mutebi *et al.*, 2001). Analysis of 42 strains isolated from humans revealed seven main genotypes, five of which occurred in Africa and two in South America. Each genotype circulated within a particular geographical region (Figure 45.12). The Angolan genotype differed greatly from all other African genotypes (17–23% nucleotide variation), suggesting that it had diverged from other East and Central African genotypes of YFV a long time ago, and that it had evolved independently. Strains that belonged to West African genotype I were genetically more heterogeneous than those of West African genotype II (Mutebi and Barrett, 2002).

The molecular-genetic findings are consistent with historical evidence that indicates that YFV originated in Africa and was, much later, carried from West Africa to South America: (i) YFV is more heterogeneous in Africa than in South America; and (ii) West African strains are phylogenetically closer to the South American strains than to strains from Central and East Africa, suggesting that South American strains evolved from West African strains.

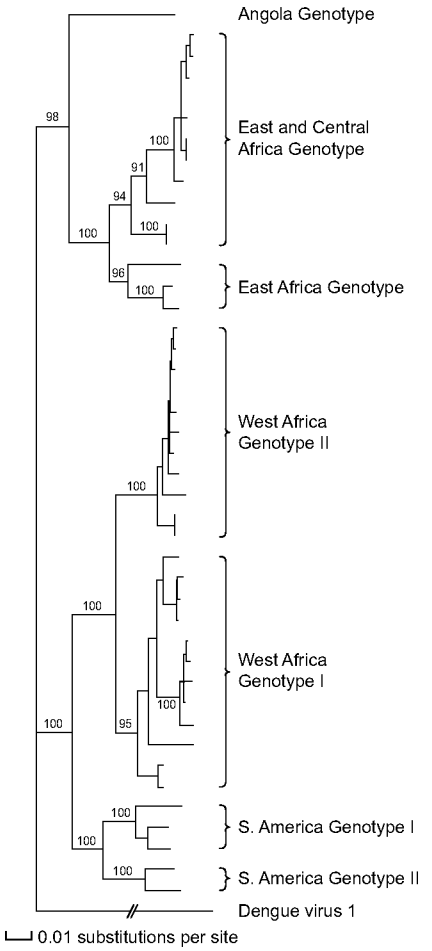

Figure 45.12 Phylogenetic relationships between seven genotypes of yellow fever virus from Africa and South America, shown in the form of a neighbour-joining tree derived from nucleotide sequences of the prM/M and E regions. (Simplified from Mutebi and Barrett, 2002.) Maximum parsimony and maximum likelihood trees had the same topology. The countries that provided strains for the seven genotypes were as follows. **Angola Genotype** – one strain from Angola. **East and Central Africa Genotype** – 11 strains from Ethiopia, Sudan, Zaire, Central African Republic. **East Africa Genotype** – three strains from Kenya, Uganda. **West Africa Genotype II** – 11 strains from Senegal, Burkina Faso, Guinea-Bissau, Ghana. **West Africa Genotype I** – 12 strains from Eastern Ivory Coast, Eastern Burkina Faso, Nigeria, Cameroon, Senegal. **South America Genotype I** – three strains from Brazil, Ecuador, Panama. **South America Genotype II** – 2 strains from Peru. The scale represents the number of genetic substitutions in analysed genomic sites on the most distal horizontal lines. The range 91 to 100 indicates relative uniformity.

(f) Extrinsic incubation period

The findings from a meta-analysis of the extrinsic incubation period (EIP) of YFV were reported by Johansson *et al.* (2010). Published records of EIP for *St. aegypti* that had fed on infected human or monkey hosts were used, and the effects of ambient temperature on the EIP were quantified. The data were fitted to four parametric survival models (exponential, gamma, Weibull, log-normal), and the relative goodness of fit of each model was assessed by the deviance information criterion (DIC). The four estimates of mean EIP (with 95% confidence interval, c.i.) of YFV in *St. aegypti* kept at 25°C were:

Exponential:	16 days (c.i. 13–20); DIC 182
Gamma:	14 days (c.i. 12–17); DIC 175
Weibull:	14 days (c.i. 12–17); DIC 173
Log normal:	12 days (c.i. 10–14); DIC 174

The Weibull model had the lowest DIC value, i.e. the best fit, and the exponential model the highest. The median EIP was shown by the Weibull model to be highly sensitive to temperature, ranging from 27 days at 15°C and 14 days at 25°C to 7.3 days at 35°C.

45.3.2 Yellow fever in Africa

(a) Introduction

Yellow fever is widespread in the Afrotropical Region, occurring between 15° N and 10° S, where regional differences in its transmission cycles can be explained in terms of vegetational zones that reflect patterns of rainfall and that govern the distribution of its primate and mosquito hosts. Yellow fever virus has never been isolated in Madagascar, which lies below 10° S. Through Central and West Africa, rainforest irregularly straddles the equator, but as the latitude increases, to north and south, rainforest is replaced sequentially by humid savannahs, semi-humid savannahs, dry savannahs and desert (Figure 45.13). In the same two directions, the length of the rainy season decreases and the tree cover progressively declines, although dense forest galleries persist into the dry savannahs. Yellow fever is enzootic or endemic in a so-called area of endemicity, which comprises rainforest and humid savannah. The northern limit of this area follows a line from 12° N in Senegal to 5° N in East Africa; the southern limit is approximately 7° S in Angola and the Democratic Republic of Congo. Because of a lack of recent data, the eastern limit is imprecise. Outside this enzootic area is an area of semi-humid and dry savannahs, the so-called area of epidemicity, which extends northwards to 15° N and southwards to 10° S. Within areas of rainforest, the virus moves through the forest and does not remain in one place. Within savannahs, YFV can remain permanently in one place and yellow fever occurs only in epidemics (Digoutte *et al.*, 1995).

Tests for specific antibodies in neural tissue or serum could detect past infections in humans or animals, and so could be used to determine the past record of yellow fever in an area. Further, by finding the youngest age group with antibodies it was possible to determine the approximate date of the last mild outbreak of a disease in the area. In the Afrotropical Region before 1940, the great majority of recorded cases of human immunity to YFV were in locations west of longitude 20° E, which at the equator is almost equidistant from the west and east coasts of the continent. From 1940 to 1951, positive cases were recorded between 20° E and 30° E, whereas most cases tested east of 30° E were negative except in parts of the Sudan and Eritrea. This suggests that YFV originated in West Africa and that during the 20th century it disseminated into eastern Africa (May, 1952).

Research on the epizootiology and epidemiology of yellow fever was undertaken earlier in East Africa than in West Africa, and investigators in the two regions conceptualized their findings somewhat differently. For that reason, East and West Africa are treated separately in later sections.

During the second half of the 20th century, apart from the Ethiopian epidemic of 1960–1962, all major epidemics reported to the World Health Organization were in West Africa. Seven occurred there between 1965 and 1987, and some of these were very severe, e.g. the epidemic in Nigeria in 1986–1987 (estimated 30,000 cases and 10,000

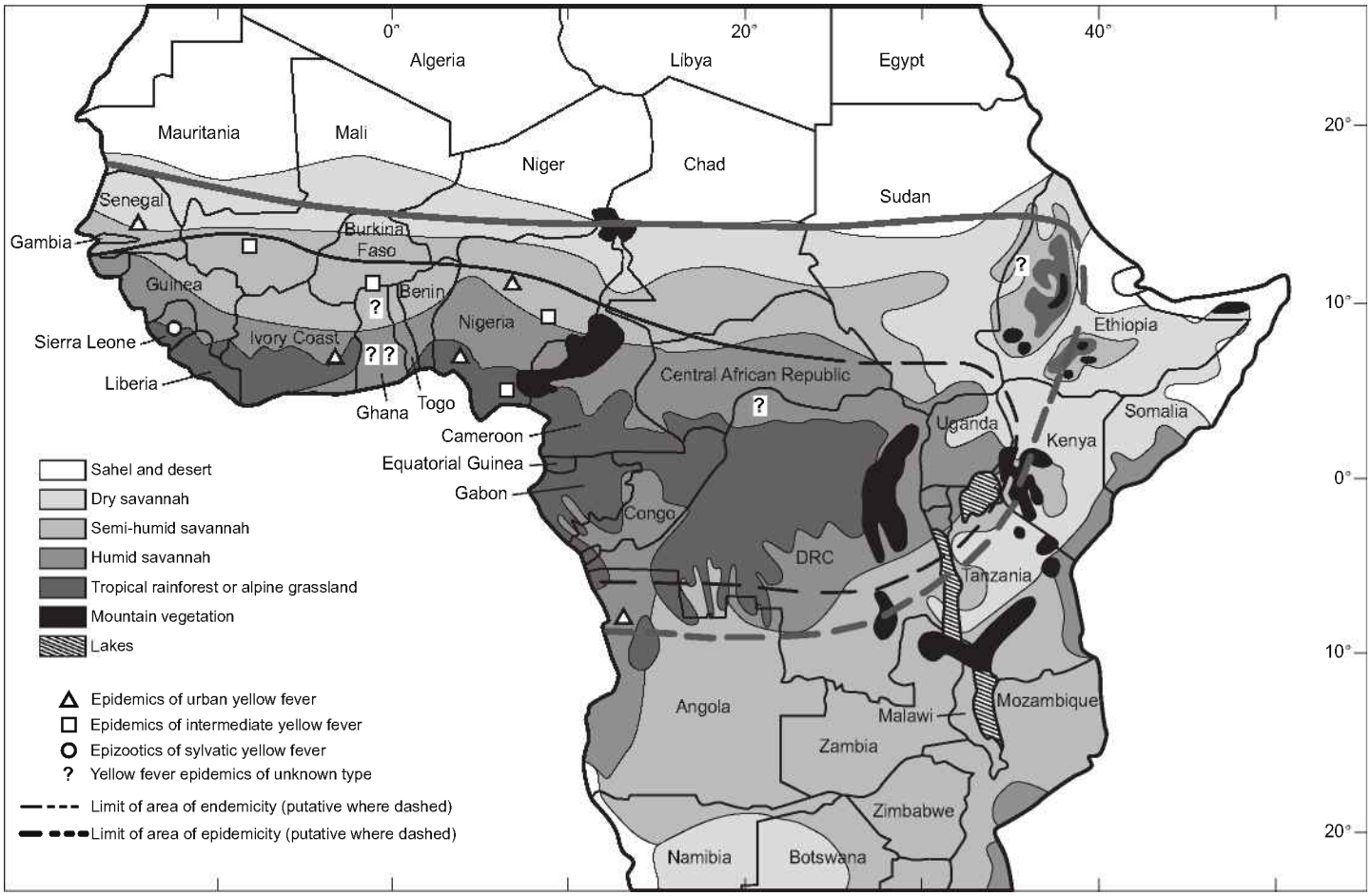

Figure 45.13 Biogeographical regions in the African continent between approximately 20° N and 20° S, characterized by habitat and vegetation types. Also marked are the regions of endemic yellow fever and potential epidemic yellow fever and the locations of the main yellow fever epidemics during the period 1960–1990. DRC, Democratic Republic of Congo. (After Digoutte *et al.*, 1995.)

deaths). Most outbreaks occurred in so-called zones of emergence in the savannah, but some occurred in urban areas through the carriage of virus by viraemic travellers (Rodhain, 1991).

(b) Mammalian hosts in Africa

Effectively, only primates are susceptible to infection. Serological and virological tests on wild-caught animals showed that YFV had infected many primates, including galagos, monkeys, apes and humans. Positive results were obtained with the following species (currently accepted binomials are used):

Order Primates
- Galagonidae
  - *Galago*: G. *senegalensis* (Senegal galago)
  - *Otolemur*: O. *crassicaudatus* (greater galago)
- Cercopithecidae (Old World monkeys)
  - *Cercopithecus*: C. *ascanius* (black-cheeked white-nosed monkey; formerly redtail monkey), C. *diana* (diana monkey), C. *mitis* (blue monkey), C. *mona* (mona monkey), C. *neglectus* (De Brazza's monkey), C. *nictitans* (white-nosed guenon)
  - *Cercocebus*: C. *torquatus* (red-capped mangabey)
  - *Chlorocebus*: C. *aethiops* (vervet monkey)
  - *Erythrocebus*: E. *patas* (patas monkey)
  - *Lophocebus*: L. *albigena* (grey-cheeked mangabey)
  - *Colobus*: C. *guereza* (guereza), C. *polykomos* (king colobus)
  - *Procolobus*: P. *pennantii* (Pennant's red colobus)
  - *Papio*: P. *hamadryas* (hamadryas baboon)
- Hominidae (great apes, humans)
  - *Pan*: P. *troglodytes* (chimpanzee)
  - *Homo*: H. *sapiens* (humans).

Systematics. Wilson and Reeder (2005). **Identification** of primate species as natural hosts of YFV: Findlay and MacCullum (1937); Haddow *et al.* (1947b, 1951); Taylor *et al.* (1955); Haddow and Ellice (1964); Monath and Kemp (1973); Germain *et al.* (1981).

A number of studies were undertaken to establish the extent of immunity to YFV in wild populations, i.e. the percentage of the population that had specific antibodies, a sign of earlier infection. Only a few studies were undertaken on galagos. At four locations in the southern Sudan, the mean proportion of *Galago senegalensis* that was immune was 1.8% (Taylor *et al.*, 1955). Near the Kenya coast, where immunity in monkeys was only 2.7%, immunity in *G. senegalensis* was 13.9% and in *Otolemur crassicaudatus* was 9.1%. In laboratory studies, YFV was transmitted to *O. crassicaudatus* by *St. africana*, and the animals showed high viraemias for 4 days (Smithburn and Haddow, 1949; Haddow and Ellice, 1964).

All African monkeys belong to the family Cercopithecidae. In the southern Sudan, immunity rates were recorded of 17% for *Papio hamadryas* (n = 94) and of 72% for *Chlorocebus aethiops* (n = 32) (Taylor *et al.*, 1955). However, in a review, Haddow (1969) stated that, in dry areas of the huge tract extending from the southern border of the Sudan to Zambia, immunity to YFV in monkeys was scarce or absent. From Ugandan lowland forest, high or moderate rates of immunity were reported in populations of six species of monkey, and low rates in one (Section 45.3.3.b). In Nigeria in the early 1970s, monkeys appeared to be restricted in both numbers and geographical range, but high percentages of some forest monkeys had specific neutralizing antibodies: *Cercopithecus nictitans*, 75%; *C. mona*, 42%; *Cercocebus torquatus*, 80%; *Chlorocebus aethiops*, 33%; *Erythrocebus patas*, 12% (Monath and Kemp, 1973).

YFV is not noticeably virulent for African monkeys and apes. Many monkeys develop an intense viraemia which lasts for some 2–5 days, making them effective amplifying hosts. Even so, infection often produces only an inapparent infection or mild clinical symptoms, such as a fever lasting 48–72 h. Monkeys characteristically live in bands, which occupy territories. The members of a band that survive infection with YFV are immune for life, so the populations are never seriously depleted; however, the number of susceptibles in a band is immediately reduced. The role that monkeys play in transmission of YFV varies with their behaviour. Different species frequent different

elevations of forest: some species stay largely in the canopy, while some others descend to ground level and may enter plantations. Savannah species usually live on the ground but sleep in trees. Wild chimpanzees can be infected, but these apes are uncommon and so are unimportant for the perpetuation of YFV (Findlay and MacCullum, 1937; Haddow *et al.*, 1947b, 1948).

(c) Mosquito hosts and vectors in Africa

Established and putative vectors:

Subfamily Culicinae; Tribe Aedini
Diceromyia
Furcifer Group: *Di. furcifer*, *Di. taylori*
Fredwardsius: *Fr. vittatus*
Stegomyia
Aegypti Group: *St. aegypti aegypti*, *St. aegypti formosa*
Africana Group: *St. africana*, *St. luteocephala*, *St. opok*
Metallica Group: *St. metallica*
Simpsoni Group: *St. bromeliae*.

Systematics. *Diceromyia*: Ferrara *et al.* (1984); Hervy *et al.* (1985); Huang (1986b); Jupp *et al.* (1993); Jupp (1998); Cook *et al.* (2005). *Stegomyia*: Huang (1979a,b, 1986a, 1988, 1990, 2004); Lutwama and Mukwaya (1994); Jupp and Kemp (1999); Mukwaya *et al.* (2000); Miyingo-Kezimbira and Lutwama (2003). *Fredwardsius*: Reinert (2000). Genera: Reinert *et al.* (2004). **Implication as vectors.** Lewis *et al.* (1942); Mahaffy *et al.* (1942); Haddow *et al.* (1948); Sérié *et al.* (1964); Haddow (1969); Germain *et al.* (1976a,b, 1981); Cornet *et al.* (1978, 1979a); Digoutte *et al.* (1995); Fontenille *et al.* (1997).

The putative vectors in the sylvatic and rural cycles of YFV are forest-dwelling aedine mosquitoes that attack primates; they include all the species listed above except *St. aegypti*. The larvae develop in tree holes and other phytotelmata, and sometimes in rock holes or artificial containers.

The Simpsoni Group comprises ten named species (Huang, 2004), of which one, identified for many years as *Aedes simpsoni*, is a main vector in outbreaks of yellow fever in East Africa. This species was thought to include populations that fed preferentially on primates or preferentially on rodents (Mukwaya, 1974; Pajot, 1977). In fact, it represented a species complex comprising *Stegomyia simpsoni s.s.*, *Stegomyia bromeliae* and *Stegomyia lilii*. In a revision of the classification of the species complex, Huang (1986a) reported that *St. simpsoni s.s.* occurred only in South Africa and Swaziland, *St. bromeliae* occurred widely in East, Central, West and southern Africa, while *St. lilii* was known from Ethiopia, Sudan and Uganda. A study of rDNA sequence divergence in internal transcribed spacer regions (ITS-1 and ITS-2) was undertaken on samples collected from wild populations at seven locations (Uganda, 5; Kenya, 1; Nigeria, 1), and which had been characterized as responsive or non-responsive to human bait in the field. A neighbour-joining tree produced two clades. All members of one clade were from locations in East Africa (Uganda, 3; Kenya, 1), and all were responsive to humans; they were identified as *St. bromeliae*. The members of the other clade were from locations in East and West Africa (Uganda, 2; Nigeria, 1), and all were non-responsive to humans; they were identified as *St. lilii*. The genetic data indicated only a weak association between the loci controlling host preference and tarsal scale pattern (which previously had been used to distinguish the species) (Mukwaya *et al.*, 2000). *Stegomyia africana* has a wide geographical distribution. It is a forest mosquito, which inhabits rainforests and the gallery forests and relict forests of the dry savannahs. As noted later, the behaviour of East and Central African populations differs from those in West Africa. *Stegomyia opok* is known from areas of savannah, where it bites in gallery forest. *Stegomyia luteocephala* is a savannah species, extending to the Sahelian zone, and occurs commonly in gallery forest and forest relicts.

Stegomyia aegypti is the vector in outbreaks of urban yellow fever. The systematics of this bitypic species, and the biology of its different forms, are described in Section 45.2.4.

For many years, two species of what was then the subgenus *Aedes* (*Diceromyia*) were putative vectors of

sylvatic yellow fever. The females of those species (*furcifer* Edwards and *taylori* Edwards) were structurally almost indistinguishable, and often specimens were identified as '*Ae. furcifer/taylori*' or as 'Furcifer Group' species. Now placed in the genus *Diceromyia*, the Furcifer Group comprises three species: *Diceromyia furcifer*, *Diceromyia taylori* and *Diceromyia cordellieri*. All three species are present in **West Africa**, where they can be distinguished by scale patterns on the scutum and abdomen and by the male genitalia. In **East Africa**, *Di. cordellieri* is known from Kenya, Tanzania and Uganda. The Furcifer Group species that occurred abundantly in southern Sudan at the time of the 1940 YF epidemic was identified by Lewis (1943) as *Di. furcifer* or *Di. taylori*, but it might have been *Di. cordellieri* (Huang, 1986b). In **South Africa**, *Di. furcifer* and *Di. cordellieri* are present. Here, the populations may be polymorphic for scale pattern, and only the male genitalia can be used for identification (Jupp *et al.*, 1993; Jupp, 1998). Now, *Di. furcifer* and *Di. taylori* can be reliably distinguished from one another by RT-PCR (reverse transcriptase PCR) (Cook *et al.*, 2005).

The Furcifer Group species are principally forest-dwelling mosquitoes, and the different species can occur allopatrically or sympatrically. *Diceromyia furcifer* and *Di. taylori* are putative vectors of YFV in West Africa. *Diceromyia taylori* is found in the canopy of forest galleries and moves only along those galleries. It is less common than *Di. furcifer* in human-bait catches, suggesting that it may have a greater preference for monkeys. In contrast, *Di. furcifer* is found in forest, at the forest edge, in open savannah, and in villages, where it attacks humans both outdoors and indoors. YFV was isolated from *Di. furcifer* and *Di. taylori* in Senegal, Burkina Faso and Côte d'Ivoire. *Diceromyia taylori* (together with *St. luteocephala*) was considered to be a main vector in the canopy of forest galleries, transmitting YFV from monkey to monkey; *Di. furcifer*, which also acts in monkey-to-monkey transmission, is the only species known to transmit to humans (Digoutte *et al.*, 1995). In the semi-humid savannahs of the northern half of Côte d'Ivoire Coast, with their dense gallery forests, *Di. furcifer* is the only species of *Diceromyia* or *Stegomyia* that has close contact with both monkeys and humans. It represented one-third of all putative vectors captured at bait in the forest (in the canopy and at ground level), and two-thirds of those captured at the forest edge or entrance to villages. Inside villages, it was almost the only putative sylvatic vector of YFV. The adults of *Di. furcifer* flew during 9 months of the year. The mean annual proportion that was parous was 55% in forest, and 70% at the forest edge and in a village (Mondet, 1997).

45.3.3 Yellow fever in East Africa

(a) Research locations

Research on yellow fever epizootiology and epidemiology was undertaken in western Uganda from 1925 onwards, and most intensively between 1937 and 1970. The principal research location was within an Administrative Division called Bwamba County, lying between the Ruwenzori Range and, to the west, the Semliki River, which at that time formed part of the boundary between Uganda and Congo (more recently called Zaire and now Democratic Republic of Congo, DRC) (Figure 45.13). On the west side of the Semliki River was the Watalinga District of the now DRC. The study area consisted of a densely populated agricultural zone and a large belt of uninhabited rainforest, all in what was termed 'lowland country', most of it at an altitude of 750 to 1000 m.

Most observations were made in the Semliki Forest, an area of primary rainforest which was an eastern extension of the great Ituri Forest of DRC. The flora and vertebrate fauna were predominantly West African, and the fauna was typical of the zoogeographical area known as the Lower Guinea Forest District. Primates recorded from Bwamba included a loris (Lorisidae) and a galago, but more importantly 11 species of monkeys and one ape, which can be assigned to the following currently recognized genera: *Cercopithecus*, 5; *Chlorocebus*, 1; *Lophocebus*, 1; *Papio*, 1; *Colobus*, 2, *Procolobus*, 1; and *Pan*, 1. Semliki Forest had been declared a sleeping-sickness area and was closed to human occupation,

consequently the forest proper was uninhabited except for a small number of nomadic pygmies. However, in Bwamba, Africans lived in scattered huts and tended plantations close to projections of the forest, while in Watalinga District villages were literally carved out of the forest. Twenty-four-hour catches were made at three heights in the forest at Mongiro and Mamirimiri (Mahaffy *et al.*, 1942; Haddow, 1945a; Haddow *et al.*, 1947a,b).

Another research location was Zika Forest, which was only 25 ha in area but was a typical part of the almost continuous, lowland rainforest extending along the northern shore of Lake Victoria. The vertebrate fauna was extensive, and the mammal fauna included monkeys and galagos. More than 95 species of mosquitoes had been identified (Corbet, 1964; Haddow, 1964, 1969).

(b) Sylvatic cycles

Eleven species of monkeys and one ape were present in Bwamba. A part of Semliki Forest that was closely observed, about 1 mile^2 (2.59 km^2) in area, supported some 400 resident monkeys, belonging to eight species and separated into 12 bands. Each band had its own territory, within which it traversed the same tree-top routes by day and returned nightly to a sleeping area. Certain species, e.g. *Lophocebus albigena*, were restricted to very small areas; in contrast, *Colobus guereza* was widespread and abundant. All of the bands had regular 'sleeping trees' in which they took up positions for the night about 20 min before dusk, and most monkeys were asleep shortly afterwards (Haddow *et al.*, 1947b). *Lophocebus albigena* and *Colobus guereza* rarely descended to the ground; therefore, transmission of YFV occurred in the forest canopy in monkey-to-monkey cycles.

Serological studies on nine monkey species in Bwamba showed a high prevalence of anti-YFV antibodies in three species (*Lophocebus albigena*, *Colobus guereza* and *Cercopithecus ascanius*), and a moderate prevalence in three others (*Cercopithecus mitis*, *C. mona* and *Papio hamadryas*), of which only a few specimens were screened. The mean prevalence of immunity among all Bwamba monkeys was 61%; the non-immunes constituted 39% (Haddow *et al.*, 1947b). In a later study, relatively low rates of immunity were found in *Chlorocebus aethiops*, monkeys that spend much of the day on the ground but sleep in the canopy at night (Haddow *et al.*, 1951).

The susceptible monkey population of Bwamba became immunized at the rate of 27% per year. The proportion that was immune increased to almost 80% by 6.5 years of age, and approached 100% after 15 years of age. A plot of percentage immunity against the mid-points of four age classes gave a smooth curve that became zero at 0.75 years (not at the origin), suggesting that very young monkeys may have passive immunity (Figure 45.14). The presence of maternally derived immunity was confirmed by tests on *Chlorocebus aethiops* from 17 localities in Uganda, in which the immunity rates with age were: infants, 33.3%; juveniles, 0%; subadults, 41.4%; adults, 61.0%; and the 'old', 65.2% (n = 164). From the proportion of females that were mature, immunity among the newborn was estimated to be 61.9%. Field observations suggested that the passive immunity persisted for a few months only. These findings of immunity increasing with age, but the presence of substantial numbers of non-immune monkeys in the population, suggested that yellow fever was endemic among the monkeys of the Bwamba lowlands (Haddow *et al.*, 1947b; Haddow, 1952).

Studies in the forest and laboratory established that *Stegomyia africana* was the main vector in the sylvatic cycle of yellow fever virus in Semliki Forest. The climate of the lowland forests of western Uganda and eastern Congo permitted *St. africana*, the dominant arboreal culicine, to transmit YFV between monkeys throughout the year. For example, in Semliki Forest the rainfall of 125–150 cm per year was well distributed, and there was no dry season in the ordinary sense. Most of the arboreal mosquitoes in Semliki Forest blood fed by day, but *St. africana* showed a pronounced peak of biting activity in the hour after dusk, when all monkeys were in the trees and quiescent. The tendency of *St. africana* to bite only during a short period after sunset, and then predominantly in the

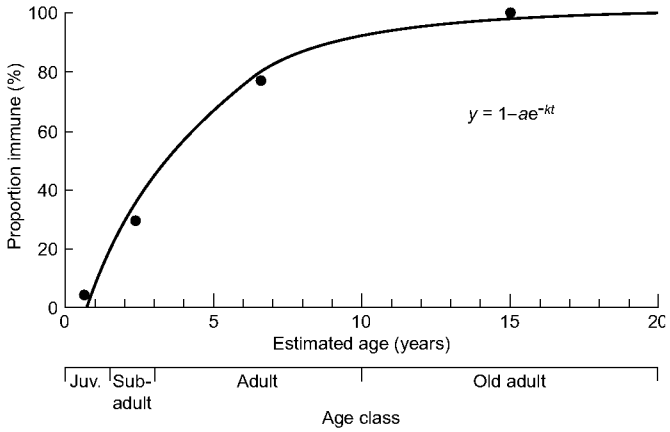

Figure 45.14 Prevalence of immunity to infection with yellow fever virus among monkeys in Bwamba, western Uganda, with respect to age, determined from the frequencies of occurrence of anti-YFV antibodies in sera from monkeys of four age classes. (After Haddow *et al.*, 1947b.) Monkeys (n = 150) of nine species were grouped into four age classes (Juvenile, Juv.; Subadult; Adult; Old adult) according to the development and wear of their teeth. Inter-species differences were insignificant, so the findings for all species were combined for further analysis. The mean number of immunes in each age class was adjusted for the frequency of that age class in the Bwamba population, and the results were plotted as percentages positive against the mid-points of the age classes. The theoretical curve was computed from the equation $y = 1 - ae^{-kt}$, in which y was the proportion positive, a (= 1.23) was derived from the constant of integration, e was the base of the natural logarithm, k (= 0.27) was the rate of increase of positives with age, and t was age in years.

lower and upper canopy at 12–24 m elevation, is illustrated by landing–biting catches on humans in Zika Forest (Table 45.7). Similar results were obtained in Semliki Forest. With anaesthetized monkeys as bait, *St. africana* formed a greater part of the total mosquito catch than with human bait. In one trial with monkey bait, the vertical distribution of landing–biting females was 0 at ground level, 44% at 10.4 m and 56% at 15.8 m. *Stegomyia africana* was the only mosquito in Semliki Forest found to harbour YFV (Haddow *et al.*, 1947a, 1948; Haddow and Dick, 1948; Haddow and Mahaffy, 1949; Ross and Gillett, 1950; Haddow, 1969). The sylvatic enzootic cycles in these lowland forests, in which YFV cycled between monkeys and *St. africana*, are illustrated in Figure 45.15.

Within the lowland forests of western Uganda there was little transmission of YFV to humans. Small numbers of pygmies lived in the forests, some of whom were seropositive for YFV, but otherwise few individuals entered the forests after dusk, and those who did were seldom attacked by the mainly canopy-dwelling *St. africana*. Some monkey species spend much time at ground level, while others will descend to ground level at the forest edge. In the Uganda lowlands, *Chlorocebus aethiops* and *Cercopithecus ascanius* commonly descend to the ground at the forest edge and raid banana plantations. Here *St. bromeliae* abounds, its larvae developing in a variety of phytotelmata, but most notably in the leaf axils of banana plants. *Stegomyia bromeliae* feeds throughout the day at heights between 0 and 5.5 m, attacking monkeys and, to a lesser extent, humans. In Uganda this was the main vector transmitting YFV from monkeys to humans. It seemed probable that, during the raids by *C. ascanius* on banana plantations and maize fields, *St. bromeliae* acquired YFV from infective individuals of that species and later transmitted it to humans working there (Figure 45.15) (Mahaffy *et al.*, 1942; Haddow, 1945b,c, 1948, 1952, 1969; Haddow *et al.*, 1947b; Smithburn *et al.*, 1949; Miyingo-Kezimbira and Lutwama, 2003).

Serological studies revealed that immunity to YFV was common among adult humans in many parts of Bwamba, and the percentage of immunes

Table 45.7 Distribution of catches of *Stegomyia africana* on human bait at ground level and at different elevations on a steel tower in Zika Forest, Uganda. (Data from Haddow, 1969; adapted from Haddow and Ssenkubuge, 1965.)

		Sunrise ← Day ← Sunset ↓	↓		*← Night →*	
Elevation		*Day*	*Hour before sunset*	*Hour after sunset*	*Night*	*Hour before sunrise*
Feet	*Metres*	*06.00 to 17.00 h*	*17.00 to 18.00 h*	*18.00 to 19.00 h*	*19.00 to 05.00 h*	*05.00 to 06.00 h*
Percentage distribution of catch with elevation in each period						
120	36.6	0	0	<1	2	0
100	30.5	<1	0	<1	3	12
80	24.4	1	1	19	12	39
60	18.3	6	19	40	55	34
40	12.2	7	27	26	21	10
20	6.1	15	27	12	4	4
0	0	71	27	3	4	2
Mean catch per hour						
All elevations		1	27	53	3	2

The elevations of the collecting platforms placed them in the following zones:
120 and 100 feet: the zone of the tallest emergent trees
80 feet: the zone of smaller emergent trees and of semi-emergent trees
60 and 40 feet: the upper and lower canopy
20 feet: a rather empty zone, just above a dense layer of shrubs and small trees
0 feet: ground; light undergrowth only, heavily shaded by a shrub–small tree layer
Because Zika Forest was only 9 miles from the equator, sunset always occurred within a few minutes of 18.00 h. The clock was always set so that 18.00 h corresponded, to the nearest minute, to the actual time of sunset (Haddow, 1964).

rose steadily as the edge of Semliki Forest was approached. During 1940–1941, a yellow fever epidemic of some magnitude occurred in the area (Mahaffy *et al.*, 1942). Surveys in western Uganda in 1946 and 1947, among children unborn at the time of an earlier mass-vaccination programme, showed immunity increasing with age and reaching 69.8% in children aged 4 years (Smithburn *et al.*, 1949). Immunity among children was largely restricted to areas adjoining the forest boundary, where both immune and non-immune *Cercopithecus ascanius* were captured (Haddow *et al.*, 1947b). It seemed likely that outbreaks of yellow fever occurred when individuals who became infected near the forest returned to nearby communities that were exposed to *St. bromeliae* (Figure 45.15).

An outbreak of yellow fever in Kenya was first noticed in September 1992; mass vaccination brought it to an end in March 1993. Human cases occurred around the headwaters of the Kerio River, Rift Valley Province, and occurred mainly in woodlands at altitudes of 1300–1800 m. Vervet monkeys and olive baboons (*Papio anubis*) were present in the outbreak area, but neither species was examined serologically for current or earlier infections with YFV. The human population of the affected area was approximately 200,000. Fifty-five cases of haemorrhagic yellow fever were identified. Most of these patients had outdoor occupations or were in regular contact with areas of dense brush. Entomological studies were undertaken in February and March 1993. The abundance of *St. africana* in that area, and two isolations of YFV from pools of that species, suggested that it had been the main vector during the main period of the outbreak. Peridomestic species were absent (Reiter *et al.*, 1998; Sanders *et al.*, 1998).

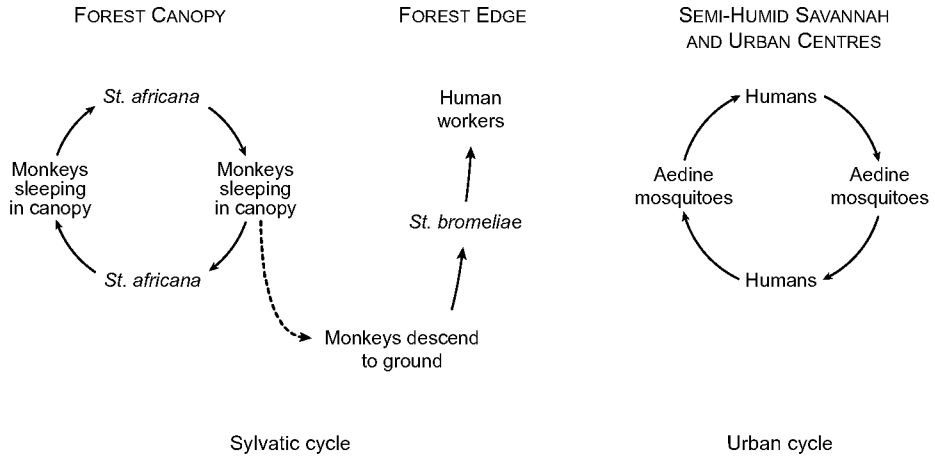

Figure 45.15 Schematic representation of the transmission cycles of yellow fever virus in East Africa. *Left*, sylvatic cycle in Semliki Forest, western Uganda, extending to human communities near the forest. In an enzootic sylvatic cycle, YFV is transmitted from monkey to monkey by the nocturnal mosquito *Stegomyia africana*. At the forest edge, monkeys of certain species often descend to the ground and raid banana plantations. There, they are attacked by the diurnal mosquito *Stegomyia bromeliae*, which can transmit YFV from monkeys to humans working in the plantations. Viraemic humans or monkeys can carry YFV to rural communities that inhabit wooded areas, where *St. bromeliae* can transmit the virus to humans. Such infections in humans are sometimes termed 'rural yellow fever'. *Right*, urban cycle in villages in the Nūba Mountains, Sudan, and in villages and towns in Ethiopia. In the Sudan, in order of importance, the putative vectors were *Fredwardsius vittatus, Diceromyia furcifer/taylori, Stegomyia luteocephala, Stegomyia metallica* and *Stegomyia aegypti*. In Ethiopia the putative vector was *St. bromeliae*. The solid arrows show the direction of virus transmission; the dashed arrow indicates movements of viraemic monkeys.

(c) Urban cycles

In certain regions of East Africa, outbreaks of YF in villages and towns had the characteristics of urban yellow fever, involving only humans as mammalian host and aedine mosquitoes as vectors. Mostly, the human communities were not situated near forest, and the putative vectors were not strictly forest mosquitoes. Major epidemics of urban yellow fever have occurred in the Sudan and Ethiopia, two adjacent countries.

In 1940, the most severe and extensive epidemic of YF recorded in Africa up to that time occurred in the Nūba Mountains of the **Sudan**. The mountains are situated between approximately 10° 30' to 12° 10' N and 29° to 31° 15' E and extend over an area of approximately 64 × 140 km. They are some 400 to 900 m higher than the surrounding plain, and consist of formations varying from isolated hillocks to ranges some 30–50 km long. Thin forest is present at altitudes of 550–600 m. Much of this area lies within the region of semi-humid savannah (Figure 45.13). The rains last from mid-May or June until October, with total rainfall varying from 650 to 990 mm. During the rains, rock pools and tree holes fill with water, producing innumerable mosquitoes, and swamps form when the water courses flood. In 1940, the human population numbered approximately 230,000, of whom 60,000 were 'Arabs' and the remainder Nūba. The wild mammal fauna included herds of baboons (*Papio hamadryas*) and of patas monkeys (*Erythrocebus patas*), while vervet monkeys (*Chlorocebus aethiops*) were generally distributed and galagos (*Galago senegalensis*) were common. YFV was known to have been present in human populations in the Nūba Mountains over many years. During 1940 there were many mild cases of presumed YF, but in ten districts in which the population size ranged from 6000 to 22,000 there were 15,267 clinical cases and 1577 deaths. The 1940 outbreak started during the rains when human movement through-

out the mountains was practically at a standstill; its sudden end was concurrent with the disappearance of mosquitoes at the end of the rains (Kirk, 1941).

Entomological investigations were started during the later stages of the epidemic. *Stegomyia aegypti* was present in only a small proportion of villages. In most dwellings water was kept in a single small earthenware pot – an unfavourable larval habitat. Larvae of *St. aegypti* were seldom found in rock holes (in fact, grinding holes), many of which were within a few feet of houses. *Fredwardsius vittatus* was very abundant during the rains, especially near the hills, where innumerable rock holes provided larval habitats. The total of 1443 mosquitoes collected while biting humans in different locations included 731 *Di. furcifer/taylori*, 461 *Fr. vittatus*, 105 *St. metallica*, 94 *St. luteocephala*, 23 *St. aegypti* and two *St. lilii*. Small numbers of *St. metallica* and *Di. furcifer/taylori* were caught on humans indoors (Lewis, 1943). No facilities were available for isolating and identifying any arboviruses in the mosquitoes.

A survey in the mid-1950s had shown **Ethiopia** to be free of YFV. In 1959, a YF epidemic reached the region of Kurmuk (10° 30' N, 34° 15' E), a large Sudanese town almost on the Ethiopian border, but it was arrested at the start of the dry season. In 1960–1962, epidemics of yellow fever spread through three epidemiologically distinct areas: (i) Sidamo Province in southern Sudan; (ii) the valley of the upper Omo River and its tributaries in south-west Sudan, a densely populated area of luxuriant, cultivated vegetation; and (iii) further north, the valley of the Didessa River, a region of dense forest and arid areas with straggly vegetation. In the forested area of Manéra near a tributary of the Omo River, the total morbidity from YF was estimated at 100,000 and the mortality at 30,000. Yellow fever virus was isolated from *St. africana* in Manéra forest, near a tributary of the Omo Rover. The virus was thought to have spread through forested areas by a sylvatic cycle involving *St. africana* and monkeys, whereas scattered human cases among men, along the Didessa River, were thought to have been acquired in the forests from bites by *St. africana*. It was surmised that the virus reached villages through visits by troops of baboons, themselves sporadically infected. YFV was isolated from a number of pools of *St. bromeliae* (cited as *Ae. simpsoni*), a species that attacked humans aggressively and that was the putative main vector (Sérié *et al.*, 1964, 1968a,b,c,d).

45.3.4 Yellow fever in West Africa – concepts

(a) Epidemiological concepts

Observations made by French investigators in West Africa during the 1970s and 1980s led them to modify the model of yellow fever epidemiology that had been developed much earlier in East Africa by Haddow and his colleagues, and they introduced new concepts concerning the ecology and transmission of YFV (Germain *et al.*, 1976b, 1978, 1981; Germain, 1984; Cordellier, 1991). In translation, these concepts may be described as follows.

(1) *Area of endemicity*: a geographical region in which YFV circulates among monkeys, being transmitted by forest mosquitoes (Figure 45.13). Both enzootic and epizootic YF occur in this area of sylvatic transmission, either continuously or in seasonal cycles. The area comprises forest (both evergreen and semi-deciduous) and regions of humid and semi-humid savannah. Rainforest is described as the 'natural focus', where YFV is maintained. (1a) A tract situated within the area of endemicity but outside the natural focus is termed the *zone of endemic emergence*. The term 'emergence' is used for any infection of human hosts due to transmission of YFV from the sylvatic circulation, and the ecological characteristics of this zone make such transmission possible. (1b) The northern limit of the area of endemicity is termed the ***endemic emergence front***. It separates the zone of endemic emergence from the area of epidemicity. Its position varies because it depends on annual rainfall patterns.

(2) *Area of epidemicity*: an area within which YFV does not circulate before an epidemic, and within which unvaccinated human populations are highly susceptible to infection. This lies within regions of

dry savannah. The term also incorporates urban centres.

The **area of endemicity**, within which YVF circulates among forest monkeys, extends from the region of equatorial forest both northwards and southwards through regions of humid and semi-humid savannah. The 'natural focus' is a region of rainforest in which YFV is conserved and *St. aegypti* is seldom or never found. The regions of humid and semi-humid savannah form the zone of endemic emergence, where transmission from the sylvatic cycle to humans is possible (Figure 45.13). Deforestation has modified the biogeographical structure of this zone. In Nigeria, by 1970, deforestation and cultivation had resulted in open terrain within the rainforest, with a predominant growth of oil palms. Farming and periodic fires had modified parts of the humid-savannah zone. Because of deforestation, the zone of endemic emergence is tending to become coterminous with the area of endemicity (Monath and Kemp, 1973; Digoutte *et al.*, 1995).

Emergences, the transmission of YFV from monkeys to humans, occur more frequently near the northern limit of the zone of endemic emergence, through the biting of species of the Africana and Furcifer Groups (Figure 45.16). In villages situated near the endemic emergence front, within areas of sylvatic transmission, outbreaks of so-called **intermediate yellow fever** may occur. These involve only the sylvatic vectors, and result from a large number of emergences, for which *Di. furcifer* is almost always responsible.

The northern limit of the area of endemicity, the endemic emergence front, is situated where continuous transmission becomes impossible. North of the endemic emergence front, in the sub-Sahelian and Sahelian savannahs, the dry season is long, there are no sylvatic cycles, and transmission from monkeys to humans does not occur. At any time, the position of the endemic emergence front

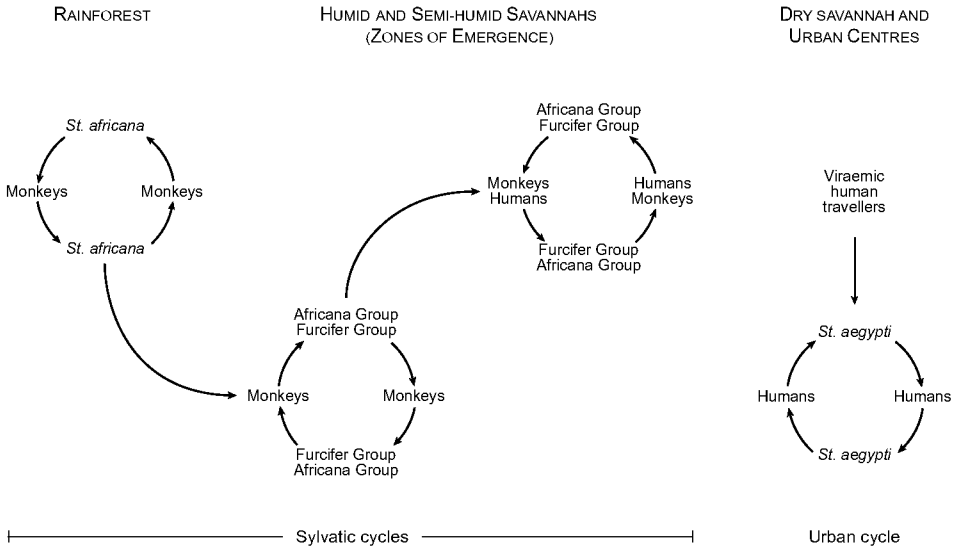

Figure 45.16 Schematic representation of the transmission cycles of yellow fever virus in West Africa. The enzootic sylvatic cycle involves forest monkeys and aedine mosquitoes of the Africana and Furcifer Groups (including *Stegomyia africana*). In zones of endemic emergence, some of the same vectors transmit YFV from monkeys to humans, causing outbreaks of yellow fever. Here, such infections in humans are sometimes termed 'intermediate yellow fever'. Movement of viraemic humans to villages in the dry savannah, or to towns or cities elsewhere, can initiate urban cycles of transmission that involve only humans and peridomestic *Stegomyia aegypti formosa*. The arrows show the direction of virus transmission.

depends on the dynamics of vector populations in the area of endemicity, which is determined by the annual patterns of rainfall. The mean latitude of the front is 11° N, but it is higher towards the west (Germain, 1984; Rodhain, 1991).

The **area of epidemicity** is heterogeneous, comprising dry and Sahelian savannahs and incorporating urban centres anywhere in the region (Figure 45.13). In the dry savannahs, the rainy season is too short to allow completion of a sylvatic amplification cycle, so maintenance of the virus is impossible. The absence of a sylvatic circulation of YFV has two consequences: only migrant humans can introduce YFV into the area; and human populations that have not been protected by vaccination are highly susceptible to infection. The area is favourable for the development of *St. aegypti*, and amplification of YFV can occur in a cycle involving humans and *St. aegypti*. When humans become infected in the zone of endemic emergence, they can carry YFV into dry savannahs where vectors with domestic or semi-domestic habits are abundant. Epidemics that occur within this area are termed **urban epidemics**, and are of two types: (i) a rural subtype, occurring in dry savannah; and (ii) an urban subtype, occurring in towns or cities. Historically, most serious epidemics have occurred within the area of epidemicity.

(b) Biogeographical regions

In West African **rainforest**, YFV exists in enzootic cycles involving monkeys and *St. africana* as the main vector (Figure 45.16). *Diceromyia cordellieri* is present, but its involvement in transmission has not been demonstrated. The monkey and vector populations are not dense, so encounters between infected vectors and susceptible monkeys are uncommon. In regions where the rainy season is long enough, transmission can take place all year round and epizootics can lead to the immunization of complete monkey bands. Consequently, the virus must pass from band to band if it is to remain in the forest. Some authors describe the rainforest as the 'natural focus' of YFV. Contact between the vectors and humans is rare, and *St. aegypti* is never or only seldom present away from human populations (Cornet *et al.*, 1977; Digoutte *et al.*, 1995).

In early descriptions of West African mosquitoes, the Simpsoni Group mosquitoes were identified as *St. simpsoni*, but were more likely to have been *St. bromeliae*. Blood-meal analysis of females captured in banana plantations in Botambi forest, near Bangui (4° 21' N, 18° 33' E), Central African Republic, showed 71.5% of meals on rodents, 21.5% on humans, 4.8% on undetermined mammals and 2.2% on birds. However, humans were seldom present in the plantations, so these figures may misrepresent host preferences (Pajot, 1977). In banana plantations in the region of Yaoundé, Cameroon, the females were highly anthropophagous (Rickenbach *et al.*, 1971).

The areas of **humid and semi-humid savannah**, which together form the zone of endemic emergence, contain many ecotones: forest/savannah mosaics; forest/savannah borders; and gallery (riverine) forests; all habitats that support concentrated populations of monkeys and vector mosquitoes. Some monkey species occupy the forested sites permanently; others use them as sleeping places. Mosquito species of the Africana and Furcifer Groups are abundant, their densities greatly exceeding those in equatorial forests. *Stegomyia africana* persists but becomes increasingly uncommon to the north, where it is replaced by *St. luteocephala*, *St. opok*, *Di. furcifer* and *Di. taylori* (Hervé *et al.*, 1976; Digoutte *et al.*, 1995).

The behaviour patterns of West African populations of *St. africana* differ from those in Uganda (where it is largely a mosquito of the forest canopy). In south-eastern Nigeria, *St. africana* is a common, sylvatic tree-hole species which frequents human dwellings, as found in two studies undertaken in the Udi Hills which surround Enugu town (6° 27' N, 7° 29' E). The vegetation was 'derived savannah forest', intermediate between the rainforest to the south and the Guinea savannah to the north. Some kilometres north of Enugu was Abor forest, an area of secondary-growth forest, lightly disturbed by human activities, with a continuous canopy at heights of 10–15 m, while to the south of

Enugu was Udi gallery forest. In human-baited collections, *St. africana* contributed >60% of all *Stegomyia* females captured. In forest, it was active at both ground level and in the canopy; in villages, it was active in compounds and to a lesser extent indoors; it was much less common at urban sites. *Stegomyia luteocephala* was active in forest and at urban sites. *Stegomyia aegypti* was most active at urban sites, but attacked at low rates in rural situations and in forest. Simpsoni Group females rarely came to human bait (Bang *et al.*, 1979, 1980). On the Jos Plateau, Nigeria, the aquatic stages of Simpsoni Group mosquitoes were commonly found, but only very few females were caught biting humans (Boorman, 1961; Lee and Moore, 1972).

In this region, mosquito densities increase with height above ground and the concentration of both monkeys and vectors in the upper canopy favours transmission (Figure 45.16). Virus amplification is affected by the susceptibility status of the monkey populations and the densities of the vector populations. The duration of epizootics – 2 or 3 years – is positively correlated with the size of the monkey population; epizootics recur at intervals of 4 or 5 years. Humans who enter the forest may become infected. Most vector species remain within the forested areas, feeding on monkeys, but certain species, such as *Di. furcifer*, can leave the forest and fly to villages, where they enter habitations. The risk of transmission to humans is high in locations where *Di. furcifer* abounds.

The regions of humid and semi-humid savannah have dry and rainy seasons, which markedly affect vector densities. Virological surveys showed that transmission of YFV increases during the rainy season, reaches a maximum during a period of about 3 months that includes the end of the rainy season and beginning of the dry season, and then declines to zero. During most of the dry season, when adult mosquitoes are absent, or present only in very small numbers, the sylvatic circulation is interrupted (Germain *et al.*, 1976b; Germain, 1984; Cordellier, 1991; Digoutte *et al.*, 1995). How YFV is maintained through the dry season is an important but unresolved question. Vertical transmission of YFV in wild mosquito populations has been reported, and its significance is discussed in Section 44.6.2.b.

The regions of **dry savannah** (Sudan savannah) that constitute the area of epidemicity are in a relatively narrow latitudinal belt that runs through Senegal, Mauritania, Mali, Burkina Faso, Niger, Nigeria and Chad, and into the Sudan, and expands widely in East Africa (Figure 45.13). Maintenance of YFV is impossible owing to the shortness of the rainy season, which prevents completion of a sylvatic amplification cycle. However, *St. aegypti* is often present where there are dwellings, its abundance favoured by domestic water storage. This is an epidemic area into which YFV is occasionally introduced by the arrival of viraemic humans, presumably from the zone of endemic emergence (Figure 45.16). There is no regular contact between the human populations and YFV, and the epidemic threat is important.

In all **urban conurbations** the high house index for *St. aegypti* constitutes a risk, because viraemic travellers may arrive from sites of YF outbreak in other towns or cities, or within the zone of endemic emergence. Humans are amplifying hosts, and, where there are large, initially susceptible populations, outbreaks of YF can be self-sustaining.

45.3.5 Yellow fever in West Africa – cases

The cases examined here illustrate some of the concepts of YFV transmission described above, and show the difficulty of obtaining sufficient evidence to implicate mosquito species as significant vectors of YFV, especially in sylvatic enzootic transmission.

(a) Sylvatic yellow fever

An investigation was carried out over several years in gallery forest in humid savannah near Kédougou, in south-eastern Senegal, following the discovery of neutralizing antibodies in young children. In 1977, intensive trapping and screening resulted in the isolation of YFV from four mosquito species, with the following MIR/1000: *Di. furcifer/taylori*, MIR = 4.08 (n = 13,479); *St. luteocephala*, MIR = 1.74 (n = 5175); *St. neoafricana*, MIR

= 5.29 (n = 189); *Fredwardsius vittatus*, MIR = 0.79 (n = 1263). When calculated per month, MIRs increased exponentially from the beginning of the rainy season up to November in the case of *St. luteocephala*, and up to December in the case of *Di. furcifer/taylori*. At the end of the rainy season, 52% of monkeys possessed neutralizing antibodies against YFV. The most common mosquito, *Di. furcifer/taylori*, was caught at elevations of 4–10 m above ground. The females were most active at dusk when the monkeys moved to the upper canopy to sleep. *Diceromyia furcifer/taylori* was considered the probable main vector of YFV to the monkeys in the Kédougou region. No YFV was isolated from 756 captured *St. aegypti*, and this was ascribed to the fact that they remained at ground level in the forest at dusk, when the monkeys were in the canopy. YFV-specific antibodies were found in a few young children, but no morbidity or mortality was noted in villages bordering the forest galleries in which YFV was circulating; it was surmised that the local strain of YFV was of low virulence to humans (WHO, 1978; Cornet *et al.*, 1979a,b).

Three factors, detailed here, were thought to affect the likelihood of a YF epizootic, the first two on the evidence that increase in the incidence of infection in monkeys with time closely followed an exponential curve. The number of infective mosquitoes becomes large enough to induce an epizootic if: either (i) the number of vertically infected mosquitoes is high at the beginning of the rains; or (ii) the rainy season is of sufficient duration. Further: (iii) The minimum number of infective mosquitoes needed to trigger an epizootic depends on the proportion of non-immune monkeys. The incidence of YF in monkeys during the summer in different forest galleries suggested that simian population densities affected the rate of amplification of YFV. Thus, in a gallery that provided sleeping trees for an important troop of baboons, the MIR was 0.59, whereas in two other galleries, which were visited only occasionally by monkeys, it was 0.22 and 0.33.

Circumstantial evidence suggested that in West Africa YFV survived between rainy seasons at foci in forested or savannah areas. For example, in forested parts of Senegal, yellow fever tended to occur in monkeys in any one place during two consecutive years; a few isolations would be recorded at the end of the transmission period of the first year and a much larger number during the second half of the transmission period of the second year. The main vectors here were *St. luteocephala* and Furcifer Group species (Cornet *et al.*, 1979a,b). Digoutte *et al.* (1995) argued that the theory of 'wandering epizootics' is not necessary to account for the perpetuation of YFV. Between epizootics, virus transmission occurs at a very low level, which is difficult to prove. However, in each year from 1970 to 1984, serological evidence was found in south-eastern Senegal of recent infection with YFV in monkeys or young children.

During 1974 and 1977, a total of 18 isolates of YFV was made from Africana Group mosquitoes caught in a gallery forest in an area of humid savannah near Bozo (5° 07' N, 18° 28' E) in the Central African Republic. Two epizootics of sylvatic yellow fever separated by a 3-year interval occurred in the small monkey population, indicating that a susceptible monkey population had been reconstituted within a relatively short period (WHO, 1978).

Outbreaks of yellow fever occurred at foci in three states in Nigeria during 1969 and 1970, when 'wild-breeding' species of *Stegomyia* were held responsible for inter-human transmission. High percentages of people inhabiting the Guinea savannah zones of the Benue and Niger River basins produced YFV-immune sera, but year-round virus transmission was considered impossible because these areas experienced a severe dry season for 5 months, when the wild *Stegomyia* populations became markedly reduced. This led to a search for regions of possible year-round maintenance of YFV, and to a detailed investigation in a remnant of gallery forest surrounding Nupeko village, south of Bida (9° 04' N, 6° 00' E) and on the upper Niger River. The mean annual rainfall at this site was 1.25 m, and during the rainy season (May to October) floods inundated the plains. Neutralizing antibodies to YFV were found in 24% of the

human population and in 57% of the monkeys tested (mostly species of *Cercopithecus*).

Larval collections produced a number of species of possible interest, including *Di. flavicollis*, *St. aegypti* and *St. luteocephala*. Between May 1971 and June 1972, in five collection periods, adult mosquitoes were collected at human or monkey bait and in light or CO_2 traps. Among 26,285 mosquitoes captured and identified to species or genus, *Mansonia africana* was the most abundant (22.4%), and members of aedine genera totalled 16.0%. However, few established or putative vectors of YFV were captured: of *Diceromyia* none; of *Stegomyia*, only six *St. aegypti* and 130 *St. luteocephala*, the latter on human bait during both wet and dry seasons. Viral assays on 23,126 mosquitoes yielded isolates of Semliki Forest virus (*Togaviridae*) and Bwamba virus (*Bunyaviridae*), but none of yellow fever virus.

The several problems addressed by these studies were not resolved. The investigators concluded that year-long, intensive and selective collection would be necessary to isolate YFV and identify a particular mosquito species as putative vector. They thought that the measured susceptibility rate of 43% among the monkeys was probably sufficient to permit continued enzootic circulation of the virus, and that mosquitoes should survive through the dry season owing to the high rainfall during the rainy season and the exceptional abundance of monkeys. Viraemic humans or monkeys might transport YFV from the gallery forest into neighbouring regions of Guinea savannah (Monath and Kemp, 1973; Lee *et al.*, 1974; Monath *et al.*, 1974).

(b) Intermediate yellow fever

The term 'intermediate yellow fever' was coined for outbreaks in West Africa that involved both monkeys and humans as mammalian hosts and both sylvan and 'domestic' mosquitoes as vectors (Cornet *et al.*, 1977). The virus could pass between monkeys and humans in either direction. The main vectors were species with 'semi-domestic' habits, largely *Di. furcifer* in northern savannah areas, and sometimes *St. africana* in moister areas. If present, *St. aegypti* might participate in human-to-human transmission. Epidemics erupted simultaneously over a wide area in certain villages but not others. The fatality rate was generally lower than in urban epidemics (Digoutte *et al.*, 1995).

During 1995, an outbreak of yellow fever occurred at a location (14° 29' N, 14° 59' W) in the Koungheul area of Senegal, when at least 46 people died among an estimated exposed population of 9000. In the villages distributed in the shrubby savannah of this so-called 'Sudanese climatic region', water for domestic use was stored both indoors and outdoors. *Erythrocebus patas* monkeys were present in the area, and the commonest mosquito was *St. aegypti formosa* in its peridomestic form. The rainy season normally lasted from July to October. In 1995, when rain last fell on 12 October, entomological surveys were undertaken between 29 October and 11 November. A total of 1125 wild-caught mosquitoes were screened for YFV, most caught responding to human bait. The following minimum infection rates per 1000 were determined: *St. aegypti* ♀♀, 54.4 (s.e. 17.2) (n = 312); *St. aegypti* ♂♂, 117.6 (s.e. 88.7) (n = 33); *Di. furcifer* ♀♀, 105.3 (s.e. 81.3) (n = 19). Respective estimated 'true infection rates' were 81.7 (s.e. 19.8), 314.2 (s.e. 156.9) and 105.3 (s.e. 76.7). Single captured females of *St. luteocephala* and *St. metallica* proved positive for YFV. Fontenille *et al.* (1997) postulated that transmission probably began with *Di. furcifer*, *St. metallica* and *St. luteocephala* as vectors. Those species disappeared soon after the end of the rainy season, when transmission continued with the only remaining vector, *St. aegypti*. The infections in male *St. aegypti* indicated a high vertical-transmission rate for YFV, and the authors surmised that vertical transmission played a major role in the 'spread' of the epidemic.

An outbreak of YF occurred in 1986 in eastern Nigeria – specifically in Oju (Benue State) and Ogoja (in the adjacent Cross River State). The affected area, lying between 6° 30' and 7° 10' N, was southern Guinea savannah, with gallery forest along streams and rivers. Here the rainy season extends from about July until October. Often, villages were situated adjacent to forest and the

villagers fetched water from streams and rivers. Domestic water storage was uncommon. The epidemic lasted from July until December, at least 6 weeks after the end of the rainy season. Oju alone probably had about 9800 cases of yellow fever with jaundice, and some 5600 deaths. Yellow fever virus was not found in the small sample of 114 *St. africana* assayed, but circumstantial evidence suggested that it was the vector: (i) the human biting rates of *St. africana* were as high as 3.5 per person per hour; and (ii) *St. aegypti* was absent from most of the area and present only at low density in part, probably because of the absence of longer-term water storage (De Cock *et al.*, 1988; WHO, 1990).

(c) Urban yellow fever

Urban yellow fever involves only humans as mammalian host and *St. aegypti* as vector. During January 1987, shortly after the outbreak of urban YF in Oju, eastern Nigeria (noted above), had ended, cases of yellow fever appeared 500 km away in Ogbomosho (Oyo State, western Nigeria), a city of 600,000 inhabitants, and within 3 weeks in several nearby towns. Probably, the virus was introduced by viraemic travellers from eastern Nigeria. The outbreak area, within the Guinea savannah vegetational zone, was one of the most densely populated in Africa, with 198 inhabitants km^{-2}. In two towns for which realistic data were obtained – Igbeti and Ejigbo, with populations of 10,000 and 30,000 – the estimated case rates were 3.5% and 6.0%, and the case fatality rates 50% and 75%, respectively. The epidemic started during the later part of the dry season. Water storage in the home was widely practised, and the large clay pots and metal drums used there provided larval habitats for *St. aegypti*. In Ogbomosho, the Breteau index (total number of containers with larvae of *St. aegypti* per 100 houses) was 676. The mean human landing–biting rate for *St. aegypti* of 2.69 per hour appeared low in relation to larval density. Yellow fever virus was not isolated from the sample of 68 females of *St. aegypti* tested, but *St. aegypti* was taken to be the vector on the grounds of its high density and the absence of other potential vectors (Nasidi *et al.*, 1989).

To investigate the vectorial competence of the Ogbomosho population of *St. aegypti*, a colony was started from eggs collected during the epidemic, and its susceptibility to infection with YFV and ability to transmit the virus to mice were measured. For comparison, colonized sylvan *St. aegypti formosa* from Oju and a colony of *St. aegypti aegypti* from Puerto Rico were submitted to the same tests. When females were fed a high dose of a strain of YFV isolated from a fatal human case during the epidemic, the susceptibility to infection of the Nigerian strains (Ogbomosho 26% and Oju 24% infected) was significantly less than that of the Puerto Rican strain (80% infected). Only 2% of Ogbomosho females and 4% of Oju females transmitted the virus to mice, whereas 34% of Puerto Rican females did so. Use of a simple mathematical model suggested that the number of bites per day needed to maintain transmission was 15 times greater for West African *St. aegypti* than for a competent strain from the Caribbean. Measurements of gene frequencies showed both Nigerian populations to be *St. aegypti formosa*. Miller *et al.* (1989) concluded that the Nigerian mosquitoes were very poor vectors of YFV, and surmised that that might be the reason why the death rates in urban yellow fever epidemics have been much lower in West Africa than in South America.

45.3.6 Yellow fever in the New World

(a) History

Yellow fever virus is one of the two flaviviruses with an Old World origin that, together with a main vector, were transported to the New World by human agency (Section 45.2.2.b). In a detailed history of yellow fever, Scott (1939) listed an outbreak of the disease in 1493 which was said to have exterminated the population of Ysabella, San Domingo, but listed no further outbreaks in the New World until one in 1620 in Cuba, which 'continued with varying intensity for the next 28 years'. The first detailed descriptions of epidemics

of yellow fever in the western hemisphere concerned an outbreak in Yucatán state, Mexico, in 1648, well over a century after the Spanish Conquest, and another in 1685–1686 at Olinda (near Recife), a seaport of northern Brazil. It is now generally accepted that YFV was carried by infected people on ships sailing from West Africa, and that *St. aegypti* was present also on those vessels, its aquatic stages surviving in water butts. At first, the trade from West Africa was conventional, but it is highly likely that during the 17th and 18th centuries the slave trade was a major factor in the transport of YFV and of its vector.

During the 17th to 19th centuries, yellow fever was a scourge of coastal settlements in South America, the larger Caribbean islands of Cuba, Jamaica, the Dominican Republic and Puerto Rico, and the West Indies – the archipelago of many small islands that extends southwards from the Bahamas to St. George's (Grenada) off Venezuela, curving arc-like around Cuba, Puerto Rico and the other large islands. From a relatively early date, ships apparently carrying YFV traded from ports in South America and the West Indies to North American coastal cities. The first reported outbreaks of yellow fever in North America were in New York (40° 41' N) in 1668, Boston (42° 20' N) in 1691, Philadelphia (40° N) in 1693 and Virginia, Minnesota (47° 31' N) in 1699 (Toner, 1873; US MHS, 1896; Scott, 1939; Digoutte *et al.*, 1995). A report prepared for the Marine-Hospital Service of the United States (US MHS, 1896) listed 448 outbreaks of YF, between 1668 and 1893 in seaports along the eastern seaboard of North America and the northern coastline of the Gulf of Mexico.

The cause of yellow fever was long unknown. J.M. Toner (1873), then President of the American Medical Association, considered that in general the savannahs and tidewater lands of the tropic and temperate zones were the most insalubrious regions, and attempted to associate particular climatic conditions with the geographical distribution of outbreaks of yellow fever in the USA. Almost 20 years later (as described in Section 45.3.1.a), through experimental investigations in Cuba by Carlos Finlay and then by Walter Reed and his colleagues, yellow fever was shown to be a mosquito-borne disease, and its vector to be *Stegomyia fasciata*, as it then became known.

The widespread distribution of *St. aegypti*, its synanthropic habits, and its high vectorial competence for YFV, made it a formidable adversary. The elimination of YF from Havana (Cuba), following attack on the aquatic stages of *St. aegypti*, inspired by Major W.C. Gorgas, led to his appointment to serve in the Panama Canal Zone, where he eventually achieved a similar success. In fact, at the Panama Canal, yellow fever proved a less refractory problem than malaria, partly because the anopheline vectors were more difficult to control, and partly because patients who recovered from yellow fever had lifelong immunity, whereas malaria patients were liable to further attacks and constituted, in some cases, a 'reservoir' of infection (Scott, 1939).

Discovery of the characteristic liver lesions of human and simian infections proved invaluable for the identification of YF cases by pathologists. The viscerotome, which could remove a sample of liver tissue post-mortem for microscopic examination, was used for many years in studies of the epizootiology and epidemiology of the disease. Its use in Brazil in the early 1930s contributed to the discovery that YF could occur in the absence of *St. aegypti* (Soper *et al.*, 1933; Elton, 1952a), and it proved invaluable in tracing the passage of yellow fever through monkey populations in South America (Soper, 1955).

YFV took hold in the New World in urban transmission cycles, with *St. aegypti* as vector and humans as amplifying hosts. When sylvatic cycles first became established is not known. A possible association between yellow fever in monkeys and humans was suggested by Balfour (1914), who had tried to follow up the assertion of elderly Trinidadians that dead and dying red howler monkeys (*Alouatta seniculus*) were always seen before outbreaks of yellow fever in humans. Much later, Davis (1930a,b,c) demonstrated transmission of YFV between monkeys by *St. aegypti*. In 1932, when there was an outbreak of yellow fever in the state of

Espirito Santo, Brazil, in the absence of *St. aegypti*, the possibility of transmission by a different vector was considered, but still supposedly between humans (Soper *et al*, 1933). Later, the term 'jungle yellow fever' was coined to describe the occurrence of yellow fever in humans who were associated with tropical forests in the absence of *St. aegypti* (Soper, 1936, 1955; Scott, 1939). Discovery of the sylvatic cycle of YF overturned the misconception that maintenance of YFV depended on just one mosquito species, introduced the concept of 'jungle yellow fever' and led to an understanding of the true source of some urban epidemics (Soper *et al.*, 1933). YFV has never been endemic in North America (Gould *et al.*, 2003).

(b) Marsupial hosts

Investigations conducted in the 1940s suggested that in Central and South America marsupials might serve as hosts and vectors of YFV:

Order Didelphimorphia (American marsupials)
Didelphidae (Opossums): *Caluromys* spp. (woolly opossums), *Didelphis* spp. (common opossums), *Philander* spp. (grey four-eyed opossums), *Metachirus* spp. (brown four-eyed opossums).

American marsupials occur abundantly in South and Central America, and were numerous in parts of Colombia where jungle yellow fever occurred persistently in the absence of adequate monkey populations. These animals produce frequent and abundant litters, continuously adding susceptible individuals to a population (Boshell, 1955).

In the Villavicencio Field Laboratory, Colombia, inoculation of five marsupial species with low doses of a local (Martínez) strain of YFV resulted in viraemias in four species (*Didelphis marsupialis*, *Philander opossum*, *Metachirus nudicaudatus* and *Caluromys philander*) and a negative result in one (*Didelphis albiventris*). The overall mean positivity rate was 16.5%. Viraemias persisted for a maximum of 4 days, with the infected animals showing no clinical symptoms. Among inoculated animals, the overall mean seropositivity rate for anti-YFV antigens was 22.8% (Bugher *et al.*, 1941). The Martínez strain of YFV was maintained in *M. nudicaudatus* for ten consecutive passes by intramuscular inoculation of serum, but without increase in virulence or infectiousness. Inoculation with an appreciable dose of virus did not always result in immunity to subsequent infection, suggesting to Bates (1944a) that susceptibility and resistance to YFV in these marsupials differed from those in primates.

Marsupials were the most numerous wild mammals in an area near Villavicencio, in eastern Colombia, where jungle yellow fever was recorded in 1934–1937. At El Horizonte, in the same region, during an outbreak of jungle yellow fever in 1940, a 24% serum positivity rate for YFV was measured in wild-caught *Didelphis marsupialis* (Bugher *et al.*, 1944). However, the serological tests might have given false positives. Sera obtained from wild *D. marsupialis* and *P. opossum* in Brazil neutralized not only YFV but also Japanese encephalitis virus, which did not occur naturally in Brazil. Koprowski (1946) concluded that the antiviral activity recorded from sera from *D. marsupialis* and *P. opossum* had been due to a 'non-specific virus-inactivating substance'.

Of 13 *Metachirus nudicaudatus* bitten by *Haemagogus janthinomys* (cited as *Hg. capricornii*) infective with YFV, 11 became infected. In contrast, only one of 11 female *Hg. janthinomys* became infected when fed on the viraemic marsupials (Bates and Roca-García, 1946a). *Haemagogus janthinomys* became infected when feeding on squirrel (*Saimiri* spp.) monkeys, but only when they circulated YFV titres in excess of 1:100,000 (Bates and Roca-García, 1946b). No marsupials had been found with titres greater than 1:1000, so it appeared that *M. nudicaudatus* and other marsupials were unlikely to be involved in natural transmission cycles with *Haemagogus*.

These early records of jungle yellow fever in localities where monkeys were very few or absent but marsupials were numerous suggest the need for reinvestigation with modern analytical techniques of the potential of American marsupials to act as amplifying hosts for YFV.

(c) Primate hosts

Order Primates:

Cebidae (New World monkeys): *Alouatta* spp. (howler monkeys), *Aotus* spp. (night monkeys), *Ateles* spp. (spider monkeys), *Cebus* spp. (capuchin monkeys), *Lagothrix* spp. (woolly monkeys), *Saimiri* spp. (squirrel monkeys)

Callitrichidae (marmosets): *Callithrix* spp., *Saguinus* spp.

Hominidae: *Homo*: *H. sapiens*.

The two families of New World monkeys are phylogenetically distinct from the Old World monkeys. They are forest-dwelling, arboreal animals. Most reports of yellow fever in New World monkeys specify the genus only, not the species. Species of *Cebus* are the most common monkeys in South America, but they are much less common in Central America, where species of *Alouatta* and *Ateles* predominate. Trinidad has just two species, *Alouatta seniculus* and *Cebus apella*.

Species of *Alouatta*, *Aotus*, *Saimiri* and *Saguinus* are very susceptible to infection with YFV, have high viraemias for a number of days, and suffer a high fatality rate. The night monkeys (*Aotus*) sleep during the day in tree hollows, so possibly have some protection from the vectors (*Haemagogus* spp.), which are diurnal and feed most readily in bright sunlight. Species of *Ateles* and *Cebus* are somewhat less susceptible. If infected, species of *Ateles* usually show high virus titres in the blood and some clinical symptoms, but seldom die; species of *Cebus* survive even better. After infection of *Callithrix penicillata* by mosquito bite, the maximum concentration of circulating virus ranged from $10^{4.3}$ to 10^{8} mouse LD_{50} ml^{-1}, but possibly those measurements were low. Species of *Lagothrix* are not easily infected, do not have high viraemias, and survive well. After an epizootic of yellow fever has passed, wave-like, through a forest, howler monkeys and marmosets will be scarce, with a small number, particularly juveniles, showing antibodies against YFV. Spider and capuchin monkeys will be almost as common as before, and a high percentage of the spider monkeys will have antibodies to YFV in their blood (Davis, 1930a,b,c; Laemmert *et al.*, 1946; Waddell and Taylor, 1946; Galindo and Srihongse, 1967; Monath, 1989; Rodhain, 1991).

Davis (1930a,b,c) reported the transmission of YFV from infective to uninfected monkeys – species of *Cebus*, *Callithrix*, *Saimiri* and *Saguinus* (cited as *Leontocebus*) – by the bites of *St. aegypti*. In the laboratory, a local strain of YFV (Rodas) was maintained for a year by mosquito–monkey passages that involved *Hg. janthinomys* (cited as *Hg. capricornii*) as vector and, sequentially, three species of monkey as vertebrate host: *Saguinus oedipus*, *Saimiri sciureus* and *Aotus trivirgatus* (Bates and Roca-García, 1946a).

(d) Mosquito hosts and vectors

Established or putative vectors:

Tribe Aedini

Haemagogus: *Hg. capricornii*, *Hg. equinus*, *Hg. janthinomys*, *Hg. leucocelaenus*, *Hg. lucifer*, *Hg. mesodentatus*, *Hg. spegazzinii*

Stegomyia: *St. aegypti*

Tribe Sabethini

Sabethes: *Sa. chloropterus*.

Classification: *Haemagogus*: Zavortink (1972); Arnell (1973); Reinert *et al.* (2004). *Sabethes*: Harbach (2011a). **Implication as vectors.** A substantial body of evidence implicates *Hg. janthinomys* as the main vector in the sylvatic cycle of YFV in the New World, and shows its involvement also in transmission from monkeys to humans. The geographical range of *Hg. janthinomys* extends from Honduras and Nicaragua through Central America, into Trinidad, West Indies, and southwards through South America as far as south-eastern Brazil and northern Argentina. A number of other species of *Haemagogus*, which have smaller geographical ranges, are putative vectors in sylvatic cycles and outbreaks of rural yellow fever. The geographical range of *Sabethes chloropterus*, another putative vector of YFV, is as extensive as that of *Hg. janthinomys*.

Here, examples are presented of the evidence that *Sa. chloropterus* and certain species of

Haemagogus meet some or all of the six requirements, listed in Section 41.1.3 and detailed below, for the identification of vectors. Some species names have been corrected according to Arnell's (1973) detailed revision of *Haemagogus*, which showed that most earlier papers that purported to describe the biology of *Hg. capricornii* or *Hg. spegazzinii falco* actually concerned *Hg. janthinomys*, a much more widespread and abundant species. For many years, only males of *Hg. capricornii* could be identified to species, from a detail of the genitalia, but Alencar *et al.* (2005) distinguished the females as that species by multivariate morphometric analysis.

(i) *The mosquito and vertebrate hosts occur sympatrically and dwell in the same habitat at the same season.* Species of *Haemagogus* and *Sabethes* have been collected at locations in forest where sylvatic yellow fever occurred among monkeys. For example, *Sa. chloropterus* and three species of *Haemagogus* (*Hg. equinus*, *Hg. mesodentatus*, *Hg. janthinomys*) were captured at sites of sylvatic yellow fever in Nicaragua (Galindo and Trapido, 1957). Isolates of YFV were obtained from pools of *Haemagogus janthinomys* and *Sa. chloropterus* collected at sites in forest in Trinidad where monkeys had recently, or shortly afterwards, died of YF (Rawlins *et al.*, 1990; Aitken and Downs, 1991; Hull *et al.*, 1991; Tikasingh *et al.*, 1991). In Brazil, *Hg. janthinomys* and *Hg. leucocelaenus* infected with YFV were captured in forest near sites where humans had been infected (Shannon *et al.*, 1938); YF-infected *Hg. janthinomys* were collected at a site of jungle yellow fever (Vasconcelos *et al.*, 2001); and YF-infected *Hg. leucocelaenus* were collected at sites where howler monkeys had died of YF and where *Hg. janthinomys* was not found (Vasconcelos *et al.*, 2003). All these mosquito species are predominantly arboreal.

(ii) *The mosquitoes feed preferentially on one or more amplifying hosts.* The forest-dwelling species of *Haemagogus* and *Sabethes* feed preferentially on primates (Galindo *et al.*, 1950).

(iii) *The infectious agent has been isolated from wild mosquitoes.* YFV has been isolated from wild mosquitoes in different regions of the New World: in Central America from pools of *Hg. equinus*, *Hg. janthinomys*, *Hg. lucifer*, *Hg. mesodentatus* and *Sa. chloropterus* (de Rodaniche and Galindo, 1957; de Rodaniche *et al.*, 1957); in Brazil from pools of *Hg. janthinomys* (Vasconcelos *et al.*, 1997, 2001); in Trinidad from pools of *Hg. janthinomys*, *Hg. leucocelaenus* and *Sa. chloropterus* (Rawlins *et al.*, 1990; Aitken and Downs, 1991; Hull *et al.*, 1991; Tikasingh *et al.*, 1991).

(iv) *The mosquito population attains a sufficient size relative to that of one or more species of amplifying host.* A number of the putative vectors have been recorded at substantial adult densities in regions of endemic yellow fever: e.g. in Panama, *Hg. equinus*, *Hg. leucocelaenus*, *Hg. lucifer* and *Sa. chloropterus* (Galindo *et al.*, 1950, 1951; Trapido and Galindo, 1956). A more useful measure is the attack rate per hour on human bait: *Hg. janthinomys*, 3-month means of up to 43.4 per man hour, in Colombia (Bates, 1944b; as *Hg. capricornii*); *Hg. equinus* 5.3 and *Hg. lucifer*, 5.2 per man hour, in Panama (Galindo *et al.*, 1950); *Sa. chloropterus*, a mean of 1.8 and maximum of 21 per man hour, in Panama (Trapido and Galindo, 1957).

(v) *In the laboratory, the mosquitoes are susceptible to infection and transmit the infectious organism efficiently.* Successful transmission of YFV from monkey to monkey has been obtained with *Hg. capricornii*, *Hg. equinus*, *Hg. janthinomys*, *Hg. mesodentatus* and *Sa. chloropterus* (Bates and Roca-García, 1945a, 1946a; Waddell and Kumm, 1948; Galindo *et al.*, 1956). *Haemagogus leucocelaenus* was somewhat more efficient than *St. aegypti* in transmitting YFV to mice; *Hg. equinus*, *Hg. janthinomys* and *Haemagogus splendens* were somewhat less efficient than *St. aegypti* (Waddell, 1949).

(vi) *The lifespan of a sufficiently high proportion of the mosquitoes exceeds the extrinsic incubation period of the virus.* Only a few studies have been made of the lifespan of species of *Haemagogus* or *Sabethes*. From measurements of the proportion that are parous, a mean daily survival rate of 0.93 was estimated for female *Hg. janthinomys* in rainforest in French Guiana (Pajot *et al.*, 1985). Use of Detinova's

technique for physiological age grading indicated daily survival rates of 0.95 and 0.96 for females of *Hg. janthinomys* in Amazonian forest in Brazil. If those values were correct, >5% of females would be alive after 2 months, and >1% after 3 months (Hervé *et al.*, 1985; Dégallier *et al.*, 1991).

Separately, on abundant evidence, *Stegomyia aegypti* has been identified as the main vector in outbreaks of urban yellow fever.

(e) Ecology of mosquito hosts

Species of *Haemagogus* occur, variably, in tropical rainforest, open deciduous forest and coastal mangrove. The species that are putative vectors of YFV are all forest-dwelling mosquitoes but differ in geographical distribution, and to some extent in habitat and seasonal abundance. For example, *Hg. janthinomys* is found almost exclusively in tropical rainforest whereas *Hg. equinus* is commonly found in rainforest, scrub and thorn forests, and mangrove, and also in peridomestic situations.

The aquatic stages of *Haemagogus* develop mostly in tree holes, but occur also in cut or broken bamboo internodes, or leaf axils of bromeliads, and occasionally in ground pools and rock holes. *Haemagogus* eggs are desiccation resistant, which ensures the survival of populations through long dry seasons. Where laid in tree holes, the eggs are deposited on moist areas above the water surface; consequently, when a tree hole dries out, eggs remain at different levels on its wall and are sequentially flooded as the tree hole refills with water. As with other aedine genera, the eggs may need a number of immersions before they hatch, a characteristic termed 'instalment hatching'. Cut bamboo internodes that had been half-filled with water and used as ovitraps for 1 year were emptied, air-dried for at least 2 weeks, and then flooded for 3 days. Repetition of this procedure led to 11 hatches of *Hg. janthinomys* eggs between 15 January and 3 August, a period of at least 31 weeks from oviposition. The largest hatch occurred after ten floodings, in mid-June, early in the rainy season (Tikasingh, 1991, 1992).

Adult *Haemagogus* are brightly coloured, day-flying mosquitoes that tend to concentrate in the upper forest canopy, where the females attack primates. *Haemagogus janthinomys* is strongly arboreal and descends to ground level only when the forest canopy has been disturbed. *Haemagogus lucifer*, *Hg. mesodentatus* and *Hg. equinus* are arboreal but less strongly so than *Hg. janthinomys*. In tropical rainforest, adults may be found during the dry season, but in greatly reduced numbers (Galindo *et al.*, 1950, 1955; Trapido and Galindo, 1956; Arnell, 1973; Tikasingh, 1991; Chadee *et al.*, 1992, 1993, 1995).

The feeding behaviour of two *Haemagogus* species was studied during a period of 27 months at a site in primary forest in French Guiana (45° 5' N, 52° 35' W). Human bait were exposed from 10.00 h to 15.00 h, the period of greatest *Haemagogus* activity, at ground level and in the canopy at 35 m elevation. *Haemagogus janthinomys* constituted 97.8% of the total catch, and *Hg. leucocelaenus* 2.2%. For *Hg. janthinomys*, the mean catch rates were 2.26 per man hour in the canopy, and 1.42 at ground level. In contrast, the mean catch rates for *Hg. leucocelaenus* were the same at ground level (0.050) and in the canopy (0.051). If the sylvatic vectors of YFV are to transmit the virus from monkeys to humans, some females must descend to and feed at ground level. The human-baited catches just noted indicate differences between *Hg. janthinomys* and *Hg. leucocelaenus* in that respect. Elsewhere in French Guiana, *Hg. janthinomys* was often captured at ground level outside human habitations, and in the surroundings of local towns (Pajot *et al.*, 1985). The catches of mosquitoes at six locations in Panama, each made at ground level and at two elevations above ground, showed substantial biting at ground level by four species of *Haemagogus* (18.4–41.7% of the total catches), and a lower rate for *Sabethes chloropterus* (9.6% of the total catch) (Table 45.8) (Galindo *et al.*, 1950).

The blood meal sources of 287 *Hg. janthinomys* caught by hand net (and so presumably at ground level) from different geographical regions of Brazil, were identified by precipitin test. Of these, 60%

Table 45.8 The vertical distribution of species of *Haemagogus* and *Sabethes* in forests in Panama as determined by landing–biting catches. Catches were made at ground level and at two higher elevations. (From the data of Galindo *et al.*, 1950.)

	Combined catches from six locations			
Species	*Ground (%)*	*Midway (%)*	*Crown (%)*	n
Hg. equinus	19.2	41.6	39.2	2370
Hg. janthinomys	18.4	30.6	51.0	351
Hg. leucocelaenus	41.7	33.0	25.3	399
Hg. lucifer	26.0	28.5	45.5	1470
Sa. chloropterus	9.6	42.4	48.1	1572

Six trapping stations were situated at altitudes between sea level and 640 m. Each station consisted of a tree to which two platforms were attached: about midway up the trunk (range 6.1–13.4 m) and in the crown (range 12.2–21.6 m). Catches were made between 08.00 and 17.30 h.

were from a single source (birds, 35.1%; rodents, 19.5%; opossums, 10.3%; humans, 12.6%; domesticated mammals, 25.2%). Of the 37% of meals from two sources, the most common combinations were bird + rodent, 16.0%; bird + human, 10.4%; and horse + human, 9.4%. Alencar *et al.* (2005) concluded that in its host responses *Hg. janthinomys* is opportunistic and adapted to a temporally and spatially variable environment.

The abundance of *Hg. janthinomys* varied seasonally, depending on rainfall. Its numbers were very low during the dry season (September–October) but it never disappeared completely (Pajot *et al.*, 1985). In quantitative studies of oviposition, ovitraps were placed in forests in Trinidad at elevations up to 8.7 m, and scored for egg numbers weekly. *Haemagogus* eggs were recovered at all elevations, and in all months of the year, but fewer traps were positive during the dry season. Tikasingh (1991) surmised that water-filled natural containers were scarce in forest during the dry season; however, gravid *Haemagogus* were present, and used the ovitraps for oviposition.

On 16 August 1954, three sick red howler monkeys were seen by woodcutters in a tree in Melajo Forest, Trinidad. One animal that fell out of the tree was captured, died during the night and was found to be infected with YFV. On 17 August, mosquitoes were collected at ground level beside that so-called 'monkey tree'; then, during a period of 8 weeks, 6435 mosquitoes were collected at human bait at the 'monkey tree' station – at ground level and from a platform at 20 m. Between 17 August and 27 September, inclusive, nine isolations of YFV were made, eight being from pools of *Hg. janthinomys* (MIR, 13.8/1000). The other isolation was from a pool of 17 other species (Downs *et al.*, 1955; Aitken and Downs, 1991).

Sabethes chloropterus inhabits both rainforest and deciduous forest. The eggs are not desiccation resistant, but they are deposited in large rot holes that are flask-shaped with a small opening and that hold water throughout the dry season. The adults are arboreal and day flying, and the females show a preference for primate hosts. In tropical rainforest they are still present, in reduced numbers, during the dry season (Galindo *et al.*, 1950, 1951, 1955; Trapido and Galindo, 1956; Chadee, 1990). In Panama, sabethines were rare during the dry season, but *Sa. chloropterus* seemed better able than species of *Haemagogus* to withstand the dry conditions (Galindo *et al.*, 1950). Evidence for the survival of adults of *Hg. janthinomys* and *Sa. chloropterus* through the dry season, and possible maintenance of YFV through the dry season, is discussed in Section 44.7.4.d. There is no evidence of vertical transmission of YFV in species of *Haemagogus* or *Sabethes*.

(f) Sylvatic cycles among monkeys

The previous sections contain information on the circulation of YFV in wild populations of monkeys of several genera in the Neotropical Region which supports the status of *Sabethes chloropterus* and certain forest-dwelling *Haemagogus* species as putative vectors. The nature of the sylvatic cycle of YFV is shown schematically in Figure 45.17.

Characteristically in the Americas, sylvatic yellow fever progresses through tropical forest as an epizootic wave. The epizootiology varies in different regions, being determined by the effects of the disease on the predominant monkey species. In Brazil, the predominant genus is *Cebus*, individuals of which very rarely die as a result of yellow fever, and movements of yellow fever through Brazilian forests were best tracked by viscerotomy of fatal human cases. In contrast, in Central America *Cebus* is relatively scarce and the predominant monkeys are species of *Alouatta* and *Ateles*, which readily succumb to yellow fever. In Central America, monkey deaths in the forest became apparent from the absence of the calls of howler monkeys (*Alouatta* spp.), and the course of epizootics could be tracked from the corpses of dead monkeys. In Trinidad, reports of dead red howler monkeys (*Alouatta seniculus*), found by individuals who had close daily contact with forests, provided the first information of outbreaks of sylvatic yellow fever. The differences in death rate suffered by different monkey genera had long-term consequences. Where monkey populations were largely or completely destroyed, development of new susceptible populations capable of supporting a later wave of infection could take many years. However, with populations of *Cebus* that survived infection, susceptible populations might reappear within a few years, able to sustain another epizootic wave (Boshell, 1955; Downs, 1955; Soper, 1955; Butcher, 1991).

During the 7-year period 1934–1940, a series of annual summer enzootics of yellow fever moved through the forested areas of south Brazil, and through Paraguay and the Misiones region of Argentina. In the course of the passage of YFV through south Brazil the invasion of towns was repeatedly observed, with outbreaks of yellow fever among the human populations due to transmission by *St. aegypti*. The source of all of these urban outbreaks was the forest; in no case was an endemic focus established (Soper, 1963).

Later, during the period 1948–1954, a wave of

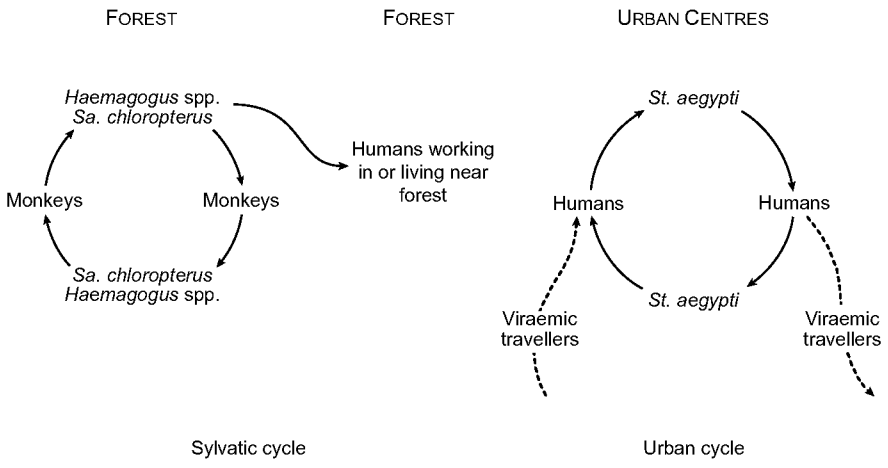

Figure 45.17 Schematic representation of the sylvatic and urban transmission cycles of yellow fever virus in Central and South America. Infections in humans who work in or live near forest are sometimes termed 'jungle yellow fever'. The single arrows show the direction of virus transmission; the dashed arrows indicate dispersion of infective humans.

sylvatic yellow fever moved slowly through the forests of Central America, from Panama to Honduras, leaving greatly reduced populations of howler monkeys. Central America is predominantly mountainous, with discontinuous lowlands on both the Caribbean and Pacific coasts flanking a highland interior. Three regions are characterized by their climate and vegetation: the interior highlands; the Caribbean plains; and the Pacific coastlands, which extend the length of the isthmus. The Caribbean plains were characterized by their tropical rainforests, which received heavy rainfall throughout the year with no marked dry season. The Pacific coastlands were covered with deciduous tropical forest, and experienced long and severe dry seasons; between November and the end of April, almost no rain fell. Since at least 1929, sylvatic yellow fever had been enzootic in an area of eastern Panama. In November and December 1948, an enzootic wave began moving westwards from that area through the Atlantic rainforest (Figure 45.18); progressing at an estimated 20–25 km per month, it reached the border with Costa Rica by June 1951. The climate of the Caribbean plains favoured persistence of vectors through the year, and the continuous progress of the epizootic wave. By 1951 and 1952 the enzootic wave advanced through Costa Rica, broadening into a front some 160 km wide before entering Nicaragua at the east and advancing towards the Isthmus of Rivas at the west. After travelling north across eastern Nicaragua, the wave moved westwards across the plains of northern Honduras to the border with Guatemala. There was some evidence from monkey deaths that the virus entered Guatemala.

In early October 1951, in southern Costa Rica, an offshoot of the wave front crossed the mountain range, the Cordillera Central, and entered the deciduous forests of the Pacific coastlands. From there, this new front moved south-east, re-entering Panama on the previously uninvolved Pacific watershed, and also moved north-west through Costa Rica. The mountain range was traversed again in June and July 1952 in northern Costa Rica, where the presence of dead monkeys on either side of the divide indicated a crossing above the 900 m contour. From the southern side, separate fronts developed during June to September 1952, one moving south-east towards Arenal Lake, a second moving through the Tempisque River Valley and south-east into the Nicoya Peninsula, and a third moving towards the Isthmus of Rivas of Nicaragua. In 1953, in the Isthmus of Rivas, the mosquito fauna seemed to disappear during the long dry season and the epizootic ended. However, as soon as the rains restarted after 5–6 months of intense drought, monkeys suddenly began to die at the exact point reached by the epizootic when the dry season had started.

In Panama, Costa Rica and Nicaragua, transmission of YFV was ascribed to *Hg. janthinomys* (cited as *Hg. spegazzinii falco*), but that species diminished in numbers as the latitude increased towards the north, and at La Ceiba in Honduras it was scarce. *Haemagogus equinus*, which occurs extensively through Central America, tolerates climatic conditions that are adverse to other species of *Haemagogus*. Many human cases of YF were recorded as the wave passed through Costa Rica, but in Nicaragua (where mass vaccination had been carried out in time) and Honduras there were relatively few human cases (Collias and Southwick, 1952; Elton, 1952a,b, 1955; Boshell, 1955).

In South America, enzootic foci of sylvatic yellow fever are found principally in the basins of the Amazon, Orinoco and Magdalena Rivers. The virus is transmitted by mosquitoes from one band of monkeys to another, and on a larger scale its movement has the form of epizootic waves that leave areas of forest either depopulated or populated with immune monkeys. Observations made during a period of 30 years at the Belem Virus Laboratory, located at the mouth of the Amazon and surrounded by heavy forest, suggested that yellow fever could be detected in the marmoset populations (*Saguinus* spp.) once every 10 years (Downs, 1982). Rodhain (1991) noted that epizootics of sylvatic yellow fever occurred in the Amazon basin every 8–10 years, but not simultaneously in its different regions. Recent ecological changes, including deforestation, expansion of human populations and restriction of monkey populations, have

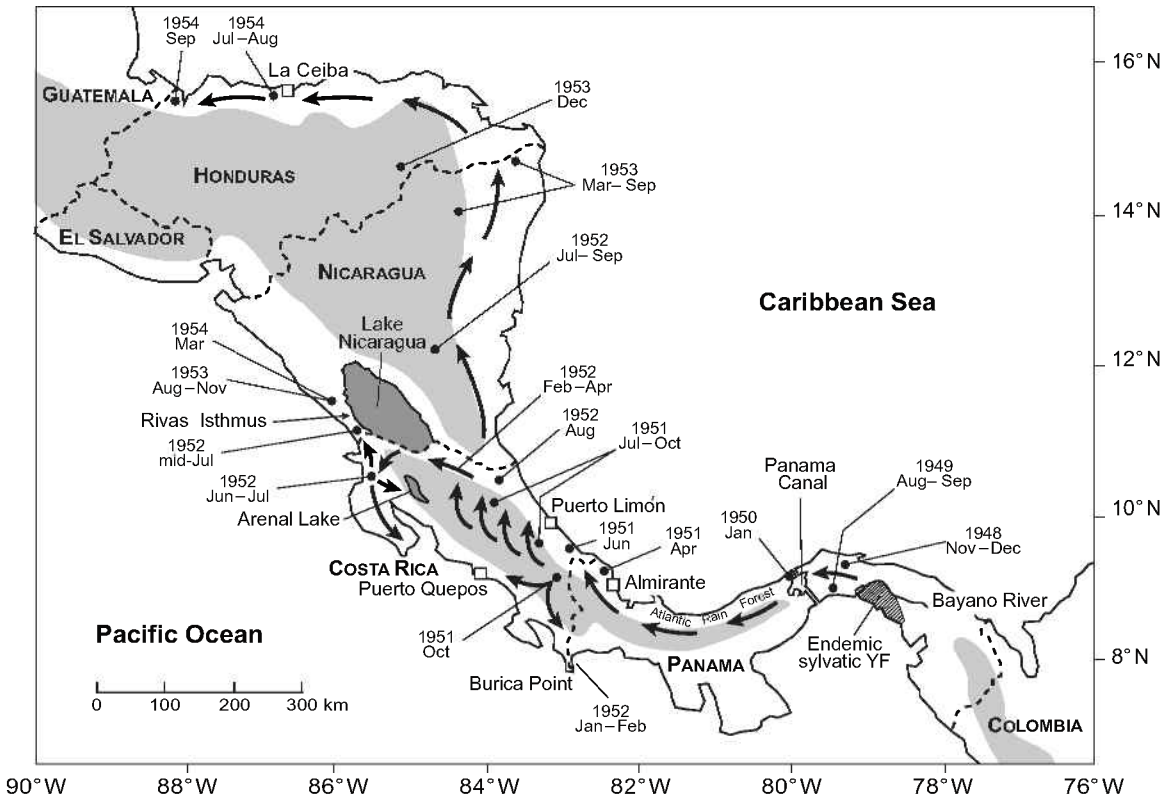

Figure 45.18 Chronology and routes of epizootic waves of sylvatic yellow fever (YF) as the yellow fever virus (YFV) moved through the forests of Central America between 1948 and 1954. In 1948, the virus emerged from an area east of the Pacific outlet of the Bayano River (see map), Panama, which had long been an area of enzootic yellow fever (since at least 1929). The subsequent routes of virus movement were revealed by the presence of sick and dead monkeys and by associated human YF cases. (From the data and maps of Elton, 1952a,b, 1955; Vargas-Mendez and Elton, 1953; Soper, 1955.) Shaded areas on the map show regions of higher altitude; →, used in the lines below to indicate places marked on the map above.

(A) Caribbean plains

Panama

1948, Nov–Dec: Panama, near Pacora, W of → Bayano River; outbreak of human YF.

1949, Aug–Sep: near Buena Vista (9° 06' N, 79° 16' W); second outbreak of human YF. Circumstantial evidence of YF epizootic in 1949 among monkeys on Barro Colorado Is. in Gatún Lake, which is part of course of Panama Canal. (Collias and Southwick, 1952.)

1950, Jan: human case from Chagres district, W of Canal Zone, and epidemic among indigenous people along the Rio Indio, slightly further west.

1951, April: near → Almirante; death of worker in virgin forest.

Costa Rica

1951, Jun: Nievecita (9° 43' N, 82° 44' W); YFV isolated from human case.

1951, Jul–Oct: along 160 km front which extended NW from → Puerto Limón towards → Arenal Lake; human YF developed explosively in five epidemic centres. Two epizootics in monkeys along this front.

1952, Feb–Apr: YF epizootic advances NW along Caribbean slope of 'continental divide', from La Fortuna towards → Isthmus of Rivas.

1952, Aug: epizootic at Pital, Alajuela (10° 27' N, 84° 17' W) and epidemic at Sarapiqui, Heredia (10° 28' N, 84° 02' W).

changed and will continue to change the distribution and intensity of transmission of sylvatic yellow fever in South and Central America (Monath, 1989, 1998).

The epizootiology of sylvatic yellow fever in Brazil during the period 1959–1999 was studied by Mondet (2001), who applied French concepts of yellow fever epidemiology (Section 45.3.4.a). Because the movements of YFV through forest were best tracked by reports of tangential (dead-end) human cases, a time–location analysis of the incidence of yellow fever in humans was used. The geographical boundaries of two main epidemiological regions were identified (Figure 45.19). (i) A **region of endemicity**, which lay within the Amazon basin. Human cases were mostly scattered and limited in number, but the region contained 'foci of endemic emergence' within which human cases were less rare, if of irregular occurrence. (ii) A **region of epidemicity**, which lay mostly outside the Amazon basin, to the north-east and particularly to the south. This region was subdivided into two parts, according to whether the outbreaks of YF were **cyclic** or **sporadic**. Parts of the region of epidemicity were forested and parts were savannah with gallery forest. All the epidemics of YF were sylvatic. In the forested regions they followed a circular path, whereas in the gallery forests of the savannah regions they followed a linear path through the narrow strips of forest. The **Pantanal** is 'the world's largest freshwater wetland system', an 'immense, pristine and biologically rich environment' (Lourival *et al.*, 2000; Swarts, 2000). Cases of human yellow fever are extremely uncommon within it, and occur only near its border (Bernard Mondet, personal communication).

During the period 1959–1999 there were three major epidemics: in 1972–1974, 1979–1982 and 1986–1992. Before each epidemic, YFV had appeared at an outbreak site in the Carajás Range,

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Nicaragua

1952, Jul–Sep: Chontales Province; active front of the main wave located within an area of approx. 40 km diameter.

1953, Mar–Sep: NE Nicaragua; human cases occur widely.

Honduras

1953, Dec: SE Honduras, Patuca and Coco valleys; monkey YF deaths and a few human cases.

1954, Jul–Aug: near → La Ceiba, northern Honduras; monkey YF deaths.

1954, Sep: near border with Guatemala; human YF case.

(B) Pacific coastlands

Southern Costa Rica

1951, Oct: valley of Cabagra River (Cabagra is 9° 09' N, 83° 11' W); many monkey deaths and one human death, showing that YFV had crossed the continental divide into the Pacific forest. Later, two trails of fatalities, in both monkeys and native farmers, began extending: one S towards → Burica Point and the other NW towards → Puerto Quepos.

1952, Feb: → Burica Point (Costa Rica/Panama); human YF fatality.

Northern Costa Rica

1952, Jun–Jul: YFV crosses the Guanacaste Mountains, E to W, to enter the Pacific forest; probably crossing above the 900 m contour between Cerro Cacao and Volcan Rincón de la Vieja (c. 10° 50' N, 85° 20' W), where dead monkeys were present on both sides side of the divide.

1952, Jun–Sep: in Pacific forests of Guanacaste Province, epizootic advances northwards towards the → Rivas Isthmus, SE towards → Arenal Lake, and SW through El Real, Guanacaste Province (10° 33' N, 85° 32' W) into the Nicoya Peninsula (not labelled).

Nicaragua

1952, mid-Jul; infected monkey in Isthmus of Rivas, just N of border with Costa Rica.

1953, Aug–Nov: Rivas Isthmus; epizootic in monkeys, ending at start of dry season.

1954, Mar: Rivas Isthmus; monkey infections resume at same place at start of rainy season.

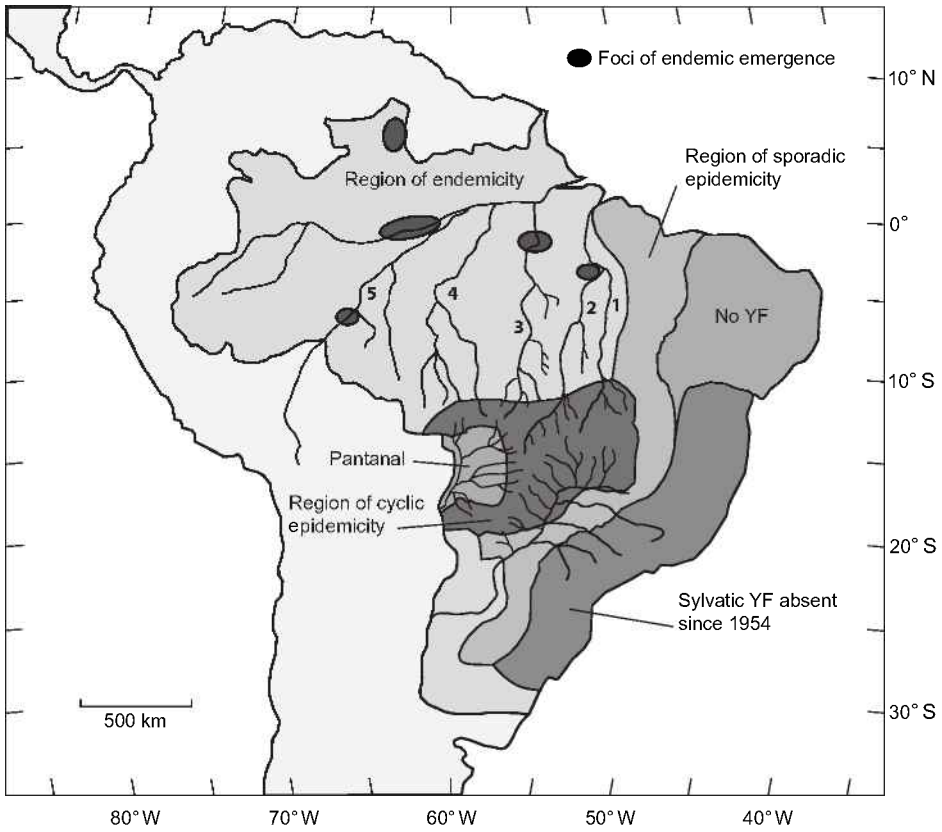


Figure 45.19 An outline map of Brazil showing the geographical boundaries of regions in which sylvatic yellow fever is endemic or epidemic. The region of epidemicity is subdivided; in one part epidemics occur sporadically, in the other cyclically. (After Mondet, 2001.) Five river courses along which yellow fever virus is thought to disperse are shown: 1, Rio Tocantins; 2, Rio Araguaia; 3, Rio Xingu; 4, Rio Tapajós; 5, Rio Maderia. This map relates to the period 1954–1999; the epidemiological regions marked on it differ from those of the 1930s, when sylvatic yellow fever was discovered.

Pará State, situated within a so-called 'area of cyclic emergence' in the region of endemicity. During a 'pre-epidemic phase', when cases were few, the outbreak followed the courses of the Tocantins and Araguaia rivers, moving southwards and upstream. Later the virus reached an 'emergence area' in the region of cyclic epidemicity, where it appeared in the form of epidemics. In this region, it followed particular south-western routes, moving from one river basin to another along the upstream courses of the rivers. The routes were almost identical in all of the three epidemics. The virus could travel several hundred kilometres within a year, or it could remain stationary for 1, 2, or occasionally 3 years, being present in an area but infecting humans seldom if at all. Outbreaks observed in the state of Rondônia State and in the north of Mato Grosso may have been caused by virus that dispersed along the rivers Xingu, Tapajós and Maderia.

Occasionally the virus left the 'area of cyclic emergence' and passed along favourable routes to reach foci in an 'area of sporadic emergence', where monkey and human populations usually were incompletely immunized against YFV, and where contact with mosquitoes was intense although restricted to the narrow strip of trees along the watercourses. There, within the states of Bahia,

Minas Gerais and São Paulo, the virus could move into other river basins. Outbreaks that occurred in human populations were preceded by epizootics which sometimes were indiscernible.

(g) Sylvatic cycles – human involvement

In 1932, cases of yellow fever occurred around the town of Santa Thereza, Valle do Chanaan, State of Espirito Santo, Brazil. The first suspected case, reported on 3 March, was fatal and the clinical diagnosis was confirmed at autopsy. Epidemiological inquiries revealed 83 suspect cases, with nine fatalities, which occurred between 15 January and 15 April 1932 scattered over a largely rural area of some 50 km^2. Probably most were cases of yellow fever, but not all. Despite intensive searches, *St. aegypti* could not be found in Santa Thereza or in the Valle do Chanaan. Thirteen towns outside the Valle do Chanaan had high indices of *St. aegypti* breeding but no suspected cases of yellow fever. After the incident in Santa Thereza, it was suggested that YFV could be transmitted between human hosts by a vector other than *St. aegypti*, probably present in the field and not 'house-limited' (Soper *et al.*, 1933). The term 'jungle yellow fever' was introduced by Soper (1936), who defined it as 'yellow fever occurring in rural, jungle and fluvial zones in the absence of *Aedes aegypti*'. He noted that the epidemiology of jungle yellow fever was quite different from that of urban yellow fever, which occurred indiscriminately among non-immunes living in or visiting infected houses, and which depended on a human–mosquito–human cycle with *St. aegypti* as the vector.

The occurrence of yellow fever among young men such as woodcutters and road workers, who disturbed the forest, led to the establishment in 1937 of a virus laboratory at Villavicencio, Meta, eastern Colombia. In 1940, scientists at the laboratory isolated YFV from *Hg. janthinomys* (cited as *Hg. capricornii*) captured in El Horizonte, not far distant. Later that year, at La Cuchilla, the nature of the contact between vector mosquitoes and woodcutters was seen by a team of entomologists and was later described by Bugher *et al.* (1944).

> On the first day of work, November 18th, 1940, an observation was made which was destined to change the entire orientation with respect to the behavior of *Haemagogus janthinomys*. In descending the mountain, the party encountered some woodcutters who were at work felling a tree. Since at this point in the forenoon, it had been noted that there were very few *Haemagogus*, the group paused to observe what would happen after the tree should fall. As the tree crashed to the ground, tearing branches from its neighbors, great numbers of *Haemagogus* were observed viciously attacking the men. It was then realized that these mosquitoes must have been present in the foliage and had been caused to fly by the disruption of their environment. It was evident that this finding, if consistent, would go far to explain the irregular catches of this species at ground level.

The association between cases of 'jungle YF' and sylvatic cycles was investigated in the following years. Research at the virus laboratory in Villavicencio revealed putative local vectors: YFV was isolated 13 times from *Hg. janthinomys* (cited as *Hg. capricornii*) and once from *Hg. leucocelaenus*. The research also revealed possible mammalian hosts: a local strain of YFV was maintained for a year by repeated mosquito–monkey passages involving *Hg. janthinomys* and local species of *Saguinus*, *Saimiri* and *Aotus* (Bugher *et al.*, 1944; Bates and Roca García, 1946a). In Brazil, of 1258 human liver specimens collected from 1932 to 1945 on which a diagnosis of yellow fever was made, only 78 came from localities where *St. aegypti* still existed. In the vicinity of Ilhéus, Bahia State, Brazil, where yellow fever was endemic, the dominant factor for immunity to YFV was contact with forests, found particularly among males over 15 years of age (Laemmert *et al.*, 1946; Taylor and Cunha, 1946).

It was not only men who entered forest who were exposed to infection; people living in rural communities situated close to forest also suffered from yellow fever. In Central and South America, during the mid-20th century, many farmers raised subsistence crops in clearings close to forest, where

exposure to YFV was a risk. The small communities were dispersed over hundreds of square miles, so access and the provision of vaccination were difficult, and cases of jungle yellow fever occurred in the working populations (Laemmert *et al.*, 1946; Taylor and Cunha, 1946; Elton, 1955; Soper, 1955).

At the present time in South America, yellow fever is regarded as a disease of human inhabitants of tropical forests, of people who go into the forests, and of people who work in clearances and plantations close to the forest edge, the virus being transmitted from simian to human hosts by native mosquitoes (Figure 45.17). In the absence of urban yellow fever from Central and South America in recent decades, we may assume that the ~3000 clinical cases reported from 1985–1999 (Monath *et al.*, 1981) were a product of sylvatic transmission cycles.

(h) Urban cycles

Epidemics of yellow fever with high fatality rates occurred in many urban centres in the New World during the 17th to the early 20th century (Section 45.3.6.a). Outbreaks were not restricted to tropical and subtropical regions but occurred in temperate North America also. They were not limited to ports but broke out far inland, probably through the carriage of infective people by the railways. To list just three of the outbreaks: in 1793, an epidemic in Philadelphia killed 5500, 10% of the population; in 1853, an epidemic caused 9000 deaths in New Orleans and a further 11,000 in the lower Mississippi Valley; in 1878, over 5000 of the 33,000 inhabitants of Memphis lost their lives to YF. The virus was highly virulent to American Indians and people of European origin, but supposedly less virulent to African slaves and their descendants (Scott, 1939; Spielman and D'Antonio, 2001).

The last epidemic of urban yellow fever in the USA occurred in New Orleans in 1905, and there has been no major urban epidemic in continental South America since 1942. During the 1950s and 1960s, an eradication campaign led to the disappearance of *St. aegypti* from most countries surrounding the Amazon basin. However, *St. aegypti* has reinvaded most major urban centres in the American tropics, and because of the low immunity of the populations, there is a renewed risk of outbreaks of urban yellow fever (Monath, 1989, 1998; Gubler, 1998). During the period December 1997 to June 1998, YF infection was confirmed in six residents of the city of Santa Cruz, Bolivia, five of whom died. Sylvatic YF was ruled out as an explanation, leading to the conclusion that these were cases of urban YF. *Stegomyia aegypti* was widespread in the city (Van der Stuyft *et al.*, 1999).

45.4 TRANSMISSION OF JAPANESE ENCEPHALITIS VIRUS

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis in humans worldwide. Japanese encephalitis (JE) is endemic in the south-east of the Russian Federation, parts of China, Korea, Japan, South Asia, South-east Asia and New Guinea. It is a leading cause of severe central nervous system infection in Asia, where 30,000 to 50,000 clinical cases are reported annually – probably an underestimate because of inadequate surveillance and reporting and because most infections are asymptomatic. About 30–35% of the clinical cases are fatal, and among the survivors 50% suffer permanent neuropsychiatric sequelae (WHO, 2010). The pathogenesis of Japanese encephalitis is outlined in Section 44.10.2.a.

JEV is a member of the clade of *Culex*-borne flaviviruses that cycle between birds and mosquitoes and can also infect mammals. The foundations of our knowledge of this zoonosis were laid in ecological and epidemiological investigations undertaken in Japan, where extensive field studies were underpinned with laboratory support. The investigations undertaken later in other countries usually addressed questions of local importance, so the early findings from Japan provide a large part of the present review.

The geographical distribution of JEV extended greatly during the 20th century, and it is still extending. Within the Asian monsoon area, the disease is endemic in tropical countries and epidemic in temperate regions and subtropical countries. The characteristics of transmission in these different regions are illustrated here with findings from temperate and subtropical regions of Japan and from a tropical region – Sarawak.

45.4.1 Japanese encephalitis virus group

(a) Species of the Japanese encephalitis virus group

Flaviviruses were formerly grouped into classes according to their serological characteristics, one such being the 'Japanese encephalitis virus serogroup' which comprised eight species (*Cacipacore virus*, *Japanese encephalitis virus*, *Koutango virus*, *Murray Valley encephalitis virus*, *St. Louis encephalitis virus*, *Usutu virus*, *West Nile virus* and *Yaounde virus*) (Fauquet *et al.*, 2005). The flavivirus serological groupings have largely been supported by later molecular-genetic characterizations, but certain inconsistencies are apparent. Phylogenetic trees derived from cDNA-sequence analysis placed St. Louis encephalitis virus (SLEV) separately in a subclade of New World bird viruses, while Cacipacore virus (CPCV), isolated from a bird in the Amazon region of Brazil, vector unknown, and the most divergent member of the group, separated with the Old World bird viruses (Section 44.1.2.b; Figure 44.2) (Kuno *et al.*, 1998; Gaunt *et al.*, 2001).

Four species of the Japanese encephalitis virus group cause serious epidemics in human populations. Three are of Old World origin: JEV itself, Murray Valley encephalitis virus (MVEV) and West Nile virus (WNV), while a fourth, St. Louis encephalitis virus (SLEV), originated in the New World. Both JEV and WNV have a propensity to disseminate. The spread of JEV into new areas is said to correlate with intensive rice cultivation

supported by irrigation (Russell, 1998; Mackenzie *et al.*, 2002a,b; WHO, 2010).

(b) Strains and genotypes of Japanese encephalitis virus

JEV was first isolated during an outbreak of encephalitis in Japan in 1935. Isolates of filterable virus from brain tissue of one encephalitic patient produced the Nakamura strain, from brain tissue of a second patient the Kamitori strain, and from spinal fluid from a third the Nakayama strain (Kasahara *et al.*, 1936). By the effects of its intracerebral inoculation into mice and by neutralization tests, the strains were distinguishable from one another and from certain known viruses (Kasahara *et al.*, 1936; Kudo *et al.*, 1937).

Okuno *et al.* (1968) used haemagglutination-inhibition and complement-fixation tests to characterize 26 strains of JEV isolated in Japan and Malaya between 1935 and 1966. The strains included three distinguishable variants of the Nakamura strain (Nakayama-Yakken, Nakayama-RFVL and Nakayama-NIH), named after the laboratories in which they were maintained. It was surmised that repeated passages through experimental animals might have led to immunological variation. Serological tests on 27 strains isolated from human tissues, pig blood or mosquito homogenates from four Asian countries showed the JEV strains to fall into at least four antigenic groups and to be distinct from the flaviviruses MVEV, DENV, SLEV and WNV (Kobayashi *et al.*, 1984). Evidence suggestive of a fifth antigenic group was provided by Kobayashi *et al.* (1985).

Later, the strains of JEV were characterized by nucleotide sequencing. This led to the characterization of four JEV genotypes, numbered I, II, III and IV by Chen *et al.* (1992), and a fifth, genotype V, proposed by Uchil and Satchidanandam (2001). The five genotypes did not correspond to the five known antigenic types. The Muar strain of JEV, which had been isolated in 1952 from a patient from Muar in Johor, Malaysia (Okuno *et al.*, 1968) was the only strain assigned to genotype V. Previously, Hasegawa *et al.* (1994) had found Muar to differ markedly from other strains in the amino acid sequences of its envelope protein.

Estimates of the mean rate of non-synonymous substitutions in the genome provide a means of determining the probable age of individual lineages. On that basis, it was determined that over 3000 years ago a lineage that gave rise to dengue virus and the JEV group of viruses diverged from the YFV lineage (Zanotto *et al.*, 1996; Gould, 2002). Similarly, Solomon *et al.* (2003) determined that genotype IV of JEV originated in the Indonesia–Malaysia region (region A in Figure 45.20) approximately 350 (± 150) years ago, diverging from a virus that was ancestral to both JEV and MVEV (its closest fully sequenced relative). Genotypes I, II and III diverged more recently, within the same region, and it was those genotypes that dispersed to other regions.

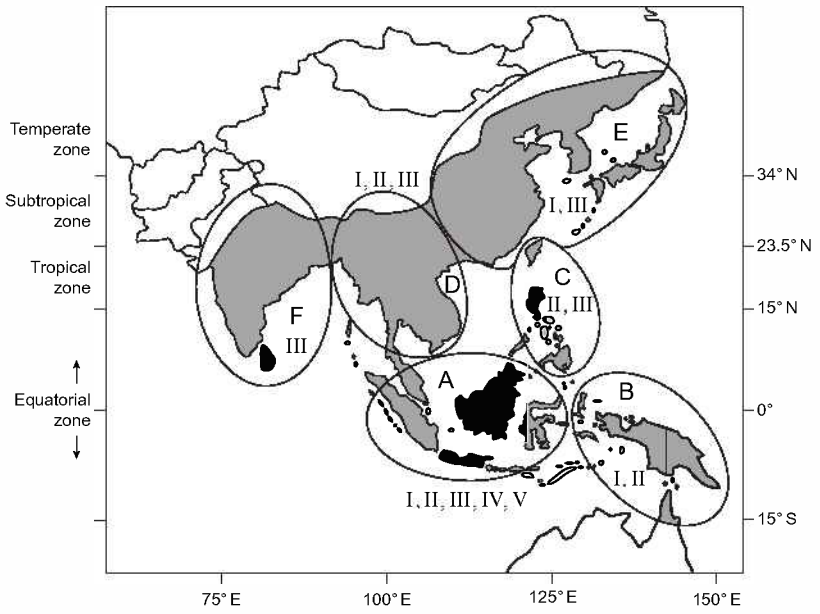
45.4.2 Geographical range and modes of dispersion of JEV

(a) Geographical range

Outbreaks of an acute febrile disease with meningitis-like symptoms and a high death rate that occurred during the summer and early autumn were described in Japan in the late 19th century. The clinical manifestations and seasonal incidence of an epidemic of acute encephalitis in Japan in 1919 showed that it differed from the epidemic encephalitis already known in Europe (Hashimoto *et al.*, 1936), i.e. 'lethargic encephalitis', first recorded in Austria in 1916 (Flexner, 1923, 1927). A large epidemic of encephalitis occurred in Japan in 1924, when >6000 cases were recorded, 60% of them fatal. During August and September 1935, epidemics of 'Type B encephalitis' broke out widely in Japan, with some 5000 clinical cases, including 1500 in Tokyo where the death rate was about 33% (Kasahara *et al.*, 1936).

The geographical range of JEV increased greatly during the 20th century, and its frontiers are still moving. The use of mice to isolate virus from clinical specimens, and the availability of characterized stocks of virus and antisera, assisted the identification of JE cases outside Japan. Outbreaks of summer encephalitis that occurred in Peking in 1935, 1938 and 1939 were later confirmed as

Figure 45.20 Distribution of reported human clinical cases of Japanese encephalitis. (Updated from WHO, 1989; Tsai *et al.*, 1999.) Six outlines superimposed on the original map delimit areas with some commonality of attributes, including temperature, rainfall and vegetation. **A.** Peninsular Malaysia, Borneo, Indonesia (part). **B.** Irian Jaya, Papua New Guinea, Cape York (Queensland, Australia). **C.** Philippines, Taiwan. **D.** Thailand, Cambodia, Vietnam. **E.** Japan, Korea, China. **F.** Nepal, India, Sri Lanka. The geographical distribution of the five Japanese encephalitis virus genotypes is indicated in Roman numerals. All five genotypes (including the two oldest, IV and V) are present in area A. The more recent genotypes, I, II and III, have dispersed from area A to other areas. (After Solomon *et al.*, 2003.)



This distributional map is presented with two reservations. (i) The records of genotype distribution are based on strains collected during a period of 60 years or more, and are not from a moment in time. During the late 20th century, in Japan, Korea and Vietnam, genotype I replaced genotype III. (ii) This map conforms to the practice of medical mapmakers whereby infections are shown as occurring throughout whole countries when they have been reported from only a part. Indonesia is an example. Rodhain (2000) commented that in most parts of Indonesia, where the inhabitants are largely Moslem, pigs are not farmed. Except for the region of Djakarta, most human clinical cases have been recorded from non-Moslem regions, including Irian Jaya and certain islands such as Bali. However, the distribution of Japanese encephalitis virus may be wider in birds than in humans. Genotype V is known only from the Muar strain, isolated only from Muar in the Malaysian peninsula, at 102° 34' E, 2° 02' N.

Japanese encephalitis by neutralization tests, and at about that time JEV was isolated in the Pacific coastal region of Russia (Innis, 1995b, review). From 1950 onwards, improved diagnostic techniques revealed the spread of the virus, but in any country the first reports of JE probably postdated the time of JEV arrival, possibly by several years. Where anti-JEV neutralizing antibodies were found first in humans, they were present also in sera from domesticated animals.

The later history of JEV dispersion has been reviewed in detail (Burke and Leake, 1988; Tsai *et al.*, 1999; Endy and Nisalak, 2002), and here just a few of the stages are noted. In Malaya, human sera that had been collected during the period 1948–1950 were found to contain anti-JEV neutralizing antibodies, and a human case of JE was diagnosed in 1951 (Paterson *et al.*, 1952). Only later was JE recognized as a serious problem in South-east Asia. In India, JE was first recognized near Vellore in Madras State in 1955 (Work and Shah, 1956), but not until 1973 was there a major outbreak. In Sri Lanka, JEV was first isolated in 1974, but the disease was only sporadic until a major outbreak in

1985 (Hermon and Anandarajah, 1974; Peiris *et al.*, 1992). By 1984, epidemics were reported from the north-east of the Indian subcontinent, Laos and Cambodia, and sporadic cases were reported from Bangladesh and Burma (Figure 45.20).

For a number of years, the known eastern limit of JE ran through Japan, the Philippines, Borneo, Java and Bali, but with an extension to the island of Guam at 144° E (Hurlbut and Thomas, 1949). Eventually, as described in Subsection 45.4.2.b below, JEV was recorded from Papua New Guinea, islands of the Torres Straits (which mostly belong to Queensland, Australia) and the Cape York Peninsula of mainland Australia (Queensland) (Figure 45.20).

With the passage of time, a genotype that is present in a particular area may be replaced. Of 11 strains of JEV isolated in Tokyo between 1935 and 1991, all were of genotype III; and genotype III was the major genotype circulating in Japan until the early 1990s. Similarly, in Vietnam, isolates of JEV obtained between 1986 and 1990 were of genotype III. Yet by the mid-1990s, strains of genotype III had disappeared from Japan and Vietnam, and strains of genotype I had supplanted them. This phenomenon was called 'genotype shift' (Ma *et al.*, 2003; Nga *et al.*, 2004); these authors surmised that genotype I was introduced from foci in South-east Asia into Vietnam and Japan, probably in the early 1990s. Analyses of nucleotide sequences in different strains of a particular genotype revealed minor but significant differences between geographically separated strains (Chen *et al.*, 1990; Hanna *et al.*, 1996; Ma *et al.*, 2003; Solomon *et al.*, 2003).

In an investigation into movements of JEV populations, 270 strains were sequenced and subjected to cladistic analysis. The phylogenetic tree obtained showed genotype I strains grouped into eight (numbered) sub-clusters. In 2001 and 2002, sub-cluster 1 strains were found in northern Vietnam but not Japan. In 2004 they were found in central China and the Mie prefecture (Honshu Island, Japan), and in 2007 in Nagasaki prefecture (Kyushu Island, Japan). The time-course suggested that strains of JEV had disseminated from Vietnam to inland China and Japan. The genotype shift that occurred in the 1990s, and the movement between 2001 and 2007 of genotype I, sub-cluster 1 strains, was consistent with the idea that strains of JEV had been reintroduced to Japan from endemic foci in South-east Asia. This concept was supported by evidence from cladistic analyses that some Vietnamese strains were phylogenetically related to Japanese strains (Nabeshima *et al.*, 2009).

(b) Extension of range to Torres Straits and Queensland

Serological evidence indicated that JEV had been present in the Western Province of Papua New Guinea (PNG) since at least 1989, while during 1997–1998 it was isolated there from *Culex* species of the Sitiens Subgroup (listed in Section 45.4.5). The isolates were most closely related to genotype II isolates from Malaysia and Indonesia (Johansen *et al.*, 2000). In 1995, three human cases of JE occurred on Badu Island in the Torres Strait – the stretch of ocean between southern PNG and the Cape York Peninsula of Queensland (Figure 45.20). Eight isolates of JEV were obtained from *Culex annulirostris* at an approximate MIR of 2.97/1000. A sentinel pig programme on islands of the Torres Strait detected JEV in every subsequent year, except 1999, up to 2005 when the programme ended. On Badu Island in 2000, one isolate of JEV was obtained from *Culex gelidus*, but none were obtained from *Cx. annulirostris* (both species of the Sitiens Group) (Hanna *et al.*, 1996, 1999; Ritchie *et al.*, 1997; van den Hurk *et al.*, 2001, 2003).

In 1998, a human case of JE and a porcine seroconversion to JEV of genotype II occurred on mainland Australia, in the Cape York Peninsula, Queensland. Six years later, during February and March 2004 and in the same area, sentinel pigs seroconverted to JEV of genotype I, after which no more seroconversions were detected. The places where JEV was detected in pigs or mosquitoes all had local concentrations of domestic or feral pigs and a high attack rate on pigs by mosquitoes of the Sitiens Subgroup. During the same year, JEV was detected in a single pool of Sitiens Subgroup mosquitoes, most of which were *Cx. annulirostris* (Hanna *et al.*, 1999; van den Hurk *et al.*, 2006, 2009a).

Two possible reasons have been proposed for the failure of JEV to disperse beyond the Cape York

Peninsula and further into mainland Australia. (i) *Preferential feeding*. Analysis of host-feeding patterns by Sitiens Subgroup species from rural locations on the western Cape York Peninsula revealed that only 5% had fed on birds and 1% on pigs, whereas 75% had fed on marsupials, probably the wallaby *Macropus agilis*. Earlier, it had been shown that two wallabies, *M. agilis* and *Macropus eugenii*, failed to develop a viraemia when infected with JEV (van den Hurk *et al.*, 2003, 2006, 2009a). (ii) *Susceptibility to infection*. Molecular diagnostic tests on *Cx. annulirostris* obtained from 43 localities in Australia, PNG and the Solomon Islands revealed five geographically restricted, and genetically divergent, lineages of the species. The southern limit of the two PNG lineages coincided exactly with the southern limit of JEV activity in the Cape York Peninsula, leading Hemmerter *et al.* (2007) to surmise that variation among the *Cx. annulirostris* lineages may explain why JEV has not yet become established on mainland Australia.

The transmission of JEV is seasonal, varies with latitude and is largely determined by climate and vector availability. In the temperate zone, vector life cycles are adapted to the annual cycle of temperature, and are regulated by day length. There, at lower latitudes the transmission season lasts from May to September, but at higher latitudes it is shorter. In warmer parts of the subtropical zone, the transmission season extends from March to late October. In the tropical zone the transmission season is determined largely by water availability, whether from monsoon rains or irrigation. Agricultural practices are a major influence on transmission because rice fields provide larval habitats for most vectors of JEV, and pigs serve as amplifying hosts. In some countries, rice-growing practices may affect larval habitats sufficiently to govern population numbers, and the regulation of pig breeding may limit the availability of susceptible amplifying hosts to particular times of the year.

(c) *Possible modes of dispersion*

The seemingly rapid extensions to the geographical boundaries of JEV during the second half of the 20th century pointed to assisted transport, of which three possible forms have been suggested: migration of amplifying hosts (especially ardeid birds); carriage on wind of infected vectors; and commercial traffic in pigs.

Ardeid birds are important amplifying hosts of JEV (Section 45.4.3). Ogata *et al.* (1970) stated, without indicating sources, that black-capped (or black-crowned) night herons (*Nycticorax nycticorax*) and intermediate egrets (*Mesophoyx intermedia*, syn. *Egretta intermedia*) migrate from Java, the Philippines, Taiwan and south China to Japan at the start of the northern hemisphere summer, and suggested that these birds may transport JEV. This hypothesis has been accepted by some reviewers (e.g. Endy and Nisalak, 2002), but perusal of the evidence fails to support it (Section 45.4.7.e). If JEV were frequently carried by viraemic migratory birds, a wider occurrence of all the genotypes might be expected than is shown in Figure 45.20.

A possible example of assisted transport over a shorter distance came with the arrival of JEV in Cape York at the northern tip of Queensland. Nucleotide-sequence homologies between isolates suggested that JEV had travelled from the Western Province of Papua New Guinea to appear in islands in the Torres Strait in 1995, and in Cape York in 1998 (cf. Figure 45.20) (Hanna *et al.*, 1996, 1999). A ban on the movement of pigs had been effectively enforced, so consideration was turned to ardeid birds as possible carriers. Many species of bird migrate annually between New Guinea and Australia, and others, including wading birds, cross the Torres Strait at irregular intervals. The rufous night heron (*Nycticorax caledonicus*), an established amplifying host, is 'nomadic' throughout the Torres Strait (Draffan *et al.*, 1983).

It was also surmised that infected mosquitoes (*Cx. annulirostris*) might be carried early in the wet season from New Guinea to islands in the Torres Strait and to Cape York by the north-west wind that usually prevails from December to April (Hanna *et al.*, 1996, 1999). Indeed, electrophoretic analyses showed that the populations of *Cx. annulirostris* in Western Province, PNG, the Torres Strait and Cape York were randomly interbreeding

(panmictic) (Chapman *et al.*, 2003). Carriage of *Cx. annulirostris* by wind had been found over New South Wales, when both males and females were trapped at altitudes between 50 and 310 m, forming 21% of the mosquito catch (n = 221) (Kay and Farrow, 2000). A simulation model of climatic conditions showed that low-pressure systems south-west of the Torres Strait produced strong northerly winds capable of carrying mosquitoes from PNG to Badu Island, and less frequently to Cape York. Backtracked simulations for earlier years showed that wind movements could account for the JE outbreaks in 1995 and 1998. However, other outbreaks had occurred in the absence of favourable winds. For example, during the period before JEV infections appeared in pigs on Badu Island in 2000, very few days had winds that could have brought mosquitoes (Ritchie and Rochester, 2001; van den Hurk *et al.*, 2001). To test for transport by wind from New Guinea, traps were operated at heights between 82 and 129 m over Saibai Island in the Torres Strait, just 4 km from the PNG coast, in January and February 2000. During a total of 30 h of northerly winds from the New Guinea mainland, small numbers of ceratopogonid flies were captured, but no mosquitoes (Johansen *et al.*, 2003).

Observations of the wind-assisted transport of *Culex tritaeniorhynchus* (also a vector of JEV) over great distances are described in Section 45.4.7.b. Human-assisted transport of JEV by viraemic birds or infected female mosquitoes is conceivable but firm evidence is lacking. Little, if anything, is known of assisted transport by viraemic pigs.

45.4.3 Avian hosts

The bird species mentioned in this section are listed in Table 45.9.

Table 45.9 Known avian hosts of Japanese encephalitis virus.

Order **Ciconiiformes**
Ardeidae
- *Ardea alba* (great egret)
- *Ardeola grayii* (Indian pond-heron)
- *Bubulcus ibis* (cattle egret)
- *Egretta garzetta* (little egret)
- *Mesophoyx intermedia** (intermediate egret†)
- *Nycticorax caledonicus* (rufous night heron‡)
- *Nycticorax nycticorax* (black-crowned night heron§)

Order **Pelecaniformes**
Phalacrocoracidae
- *Phalacrocorax carbo* (great cormorant)
- *Phalacrocorax carbo hanedae*¶ (great cormorant)
- *Phalacrocorax niger* (little cormorant)

Order **Gruiformes**
Rallidae
- *Gallicrex cinerea* (watercock)

Order **Passeriformes**
Corvidae
- *Corvus splendens* (house crow)
- *Cyanopica cyanus* (azure-winged magpie)

Passeridae
- *Passer montanus* (Eurasian tree sparrow)

Turdidae
- *Turdus naumanni* (dusky thrush)

Sturnidae
- *Sturnus cineraceus* (white-cheeked starling #)
- *Sturnus contra* (Asian pied starling)
- *Sturnia pagodarum* (Brahminy starling)

Order **Galliformes**
Phasianidae
- *Gallus gallus domesticus* (domestic chicken)

The scientific names accord with 'Avibase – the world bird database' at http://avibase.bsc-eoc.org/.
*, *Egretta intermedia* of some authors; †, plumed egret of some authors; ‡, Nankeen night heron of some authors; §, black-capped night heron of some authors; ¶, subspecies present in Japan; #, grey starling of some authors.

(a) Ardeid amplifying hosts

In Japan, findings of JEV infections in wild birds, including herons and egrets (family Ardeidae), led to the investigation of a number of ardeid populations. The aggregations of birds in heronries were amenable to screening: the nestlings were large enough to withstand repeated withdrawals of blood; and samples could be taken from many birds during short periods of time. JEV was isolated from blood samples from black-crowned night herons, intermediate egrets and little egrets (*Egretta garzetta*), and it transpired that a number of ardeid species were hosts in JEV transmission cycles (Hammon *et al.*, 1958; Buescher *et al.*, 1959b; Scherer and Buescher, 1959).

Up to 2–3 weeks of age, nestling ardeids were covered only by loose down, which was little impediment to mosquito feeding. After 3–4 weeks of age, feathers covered the skin except on the legs and near the eyes, and provided some protection. Adult herons fed at night, egrets during the day. Consequently, at dusk and dawn, the principal periods of mosquito feeding, the egret nestlings were usually covered by adult females, whereas heron nestlings were alone in their nests. Nests at low elevation (1–2 m) were attacked by more mosquitoes than nests at higher elevation (8–18 m) (Scherer *et al.*, 1959b).

Sufficient evidence was obtained to prove that certain ardeids, notably the black-crowned night heron, were amplifying hosts of JEV: (i) there was a high incidence of natural infection, with development of viraemia; (ii) wild *Cx. tritaeniorhynchus* fed in substantial numbers on these birds; (iii) the viraemias lasted long enough and in titres adequate to infect colonized *Cx. tritaeniorhynchus*; (iv) colonized females of *Cx. tritaeniorhynchus* transmitted the virus from infective to susceptible herons; and (v) each year large populations of susceptible ardeids were replenished (Gresser *et al.*, 1958; Scherer *et al.*, 1959c).

Subcutaneous inoculation with JEV of black-crowned night herons, intermediate egrets and little egrets led, 2–4 days later, to viraemias of $10^{4.2-4.7}$ $MICLD_{50}$ (mouse intracerebral LD_{50} ml^{-1}) which lasted for 2–5 days. Black-crowned night herons were distinctly more susceptible than little egrets to infection from a given dose (Buescher *et al.*, 1959d). Experimental inoculation of rufous night herons led to viraemias of $10^{1.9-4.8}$ mouse LD_{50} ml^{-1} (Boyle *et al.*, 1983). The amount of virus circulating in the blood of experimentally or naturally infected ardeids was sufficient to infect females of *Cx. tritaeniorhynchus* that fed on them (Gresser *et al.*, 1958).

Anti-JEV HI antibody appeared in black-crowned night herons, little egrets and intermediate egrets within 2–3 days of cessation of viraemia, and reached maximum titres within 14–21 days of infection. The titre usually decreased during the second and third months post-infection. Neutralizing antibody appeared 3–12 weeks post-infection, and by 9 months post-infection showed little evidence of loss (Buescher *et al.*, 1959d). In field studies, the HI test indicated the time and incidence of natural infection more accurately than virus isolation studies, because the antibody persisted longer than the viraemias (Buescher *et al.*, 1959b).

It was known that antibodies passed from hen birds to their chicks. A search for anti-JEV antibodies showed neutralizing antibodies and HI antibodies to be present in wild nestlings of black-crowned night herons (30% and 10%, respectively), of intermediate egrets (28% and 2%) and of little egrets (9% and 0) (n >500). The low percentages in little egrets accorded with the lower natural infection rate in that species. Neutralizing antibodies were present in 31% of Japanese cormorant (*Phalacrocorax capillatus*) nestlings. Maternal antibody present in nestlings of black-crowned night herons prevented infection by JEV inoculation. However, the maternal neutralizing antibody disappeared with age, and by 30 days no intermediate egrets and very few black-crowned night herons possessed it. Once maternal antibodies had disappeared, the young ardeids were susceptible to infection with JEV (Buescher *et al.*, 1959e). Owing to their relatively rapid disappearance from ardeid nestlings, maternal antibodies would have had relatively little influence on wider JEV transmission,

but within heronries the ecology of JEV would have been affected by the antibody production that followed natural infection, with an increasing proportion of birds becoming immune with age (Buescher *et al.*, 1959a,b; Scherer *et al.*, 1959a,b).

In India also, JEV infects ardeids. For example, in a delta between the Krishna and Godavari rivers, anti-JEV neutralizing antibody was found in 35% of Indian pond-herons (*Ardeola grayii*; *n* = 285), and 34% of cattle egrets (*Bubulcus ibis*; *n* = 229). Because 72% of the infected birds were less than 6 weeks old, it was suspected that in most of them the neutralizing antibody was of maternal origin (Rodrigues *et al.*, 1981).

(b) Other birds

The findings from an early study in Japan suggested that birds of many species had been infected with JEV. Over 20% of blood samples taken from shot juveniles or adults of 154 bird species were positive for anti-JEV neutralizing antibodies, leading Hammon *et al.* (1958) to conclude that 'birds in large numbers' were infected with JEV and 'might serve as an important source of mosquito infection'. Other workers, who used nets to capture five species of migrating thrushes (*Turdus* spp.) listed in an earlier study, failed to confirm the findings, and this led them to re-examine the sampling procedures. Near Tokyo, blood samples were taken from passerine birds of four different genera: some birds of each species were shot in flight and then bled; others were netted and bled while alive. Samples from many of the shot birds gave false-positive scores (Table 45.10) (Scherer *et al.*, 1964). Further analyses of the same blood samples suggested that specific and non-specific neutralizing antibodies had been released into the plasma of the shot birds, possibly due to trauma to abdominal and thoracic viscera (Hardy *et al.*, 1964). The early findings of Hammon *et al.* (1958) have been cited without qualification by other authors, so it is necessary to emphasize the need for caution in serological studies of wild birds.

Records of JEV infections in non-ardeid birds are rather sparse. In Japan, anti-JEV neutralizing antibody of maternal origin was found in five of 16 great cormorant nestlings (*Phalacrocorax carbo hanedae*; Phalacrocoracidae), 2–4 weeks of age (Buescher *et al.*, 1959e). In a delta area in south India, anti-JEV neutralizing antibody was present in 10% of little cormorants (*Phalacrocorax niger*) (*n* = 31) trapped as young or adult birds (Rodrigues *et al.*, 1981). Anti-JEV neutralizing antibody was found, but only very rarely, in azure-winged magpies (*Cyanopica splendens*, Corvidae) in Japan (Buescher *et al.*, 1959b). Neutralizing antibodies were found in 20–37% (*n* = 116) of Eurasian tree sparrows (*Passer montanus*; Passeridae) netted on Kyushu. Uninfected sparrows inoculated with JEV developed viraemias (Hasegawa *et al.*, 1975).

Japanese encephalitis was first recorded in an area near Vellore, South India, in 1955, but there was no rapid spread of JEV to birds. Many blood samples were taken from birds caught in that area during 1962 and 1963, and to a much lesser extent from 1964 to 1966, and tested for JEV or anti-JEV neutralizing antibody. Of 1396 blood samples taken from mostly adult birds of 71 species (including five species of ardeids), 19 contained virus but not JEV. Of 410 blood samples taken from 52 species of birds (including three species of ardeids), only two passeriforms (Brahminy starling,

Table 45.10 Anti-JEV neutralizing activity in blood samples that had been taken from either live-caught birds or birds that had been shot. (From Scherer *et al.*, 1964.)

Species	*Number of birds with LNI* > 1.6*	
	Live caught; jugular venepuncture	*Shot; cardiac puncture*
Dusky thrush	1 (87)†	17 (53)
Azure-winged magpie	0 (25)	19 (41)
Eurasian tree sparrow	1 (77)	21 (40)
White-cheeked starling	0 (37)	3 (28)
Totals	2 (226)	60 (162)

*, LNI = $\log_{10}$ neutralization index; †, in parentheses, numbers of birds tested.
See Table 45.9 for Latin names of bird species.
Blood samples were taken by jugular venepuncture from live birds captured by mist netting, and by cardiac puncture from birds within 2–10 min of their being shot. Results were assessed from the proportion of samples with LNI > 1.6.

Sturnia pagodarum; house crow, *Corvus splendens*) were positive for anti-JEV neutralizing antibody (Carey *et al.*, 1968).

The development of viraemia in fowl chicks and ducklings after inoculation with JEV, and the transmission of virus by mosquitoes from infective to non-infected chicks or ducklings (Buescher *et al.*, 1959d; Dhanda *et al.*, 1977), raised the possibility that these domesticated birds might serve as amplifying hosts.

45.4.4 Mammalian hosts

Wild mammals of several taxonomic orders have been shown to contain anti-JEV antibodies, and so had been exposed to the bites of infective vectors, but only relatively few species develop a marked viraemia after infection.

(a) Pigs

Sus scrofa (Suidae) occurs widely through the world, in populations of feral wild boars and as domesticated pig varieties. In most of Asia, farmed pigs are the main amplifying host of JEV. During a period of approximately 2–6 days after inoculation with JEV, pigs developed viraemias of 10^4–10^6 mouse LD_{50} ml^{-1} blood. By the time the period of viraemia ended, IgM had appeared and was increasing in titre. Pigs infected with JEV usually do not show any clinical symptoms (Scherer *et al.*, 1959g; Kodama *et al.*, 1968; Hayashi *et al.*, 1976). Depending on husbandry practices, new litters may be produced at any time of year, and typically the pigs are slaughtered at about 6–8 months of age. Because of the rapid turnover of pig populations, these animals can be an important source of mosquito infection. Often, pigs are penned in close proximity to human dwellings.

That farmed pigs were important amplifying hosts of JEV was established by the following lines of evidence: (i) there was a high incidence of infection, with development of viraemia; (ii) the viraemias lasted long enough and in titres adequate for experimental infection of *Cx. tritaeniorhynchus*; (iii) colonized females of *Cx. tritaeniorhynchus* transmitted the virus from infective to uninfected pigs; (iv) wild *Cx. tritaeniorhynchus* showed a strong propensity to bite pigs; and (v) each year large populations of susceptible pigs were replenished (Gresser *et al.*, 1958; Scherer *et al.*, 1959c).

(b) Other domesticated mammals

When infective mosquitoes feed on other domesticated animals, the result in most species is an inapparent infection manifested by an immune response only.

In Japan, several hundreds to thousands of cases of equine JE were reported annually through the 1930s and 1940s, prior to the introduction of equine vaccination in 1948 (Konishi *et al.*, 2004). Serological studies in Beijing (1951–1954) revealed high percentages of anti-JEV neutralizing antibodies in domesticated animals: horses, 94%; donkeys, 94%; cattle, 92%; and dogs, 66%. Of these, only horses and donkeys might develop encephalitis (Huang, 1982). Serum samples from domesticated animals in different locations in Malaysia showed the following prevalences of anti-JEV HI antibodies: in cows, 11–75%; buffalo, 28–63%; goats, 6–26%; and sheep, 1–33% (Oda *et al.*, 1996). When four calves received multiple bites from JEV-infected *Cx. tritaeniorhynchus*, none became viraemic, but all developed neutralizing antibodies to JEV at 21–65 days post-infection (Ilkal *et al.*, 1988). In contrast, horses infected with JEV by mosquito bites developed viraemias which lasted for 3 days, and JEV could be transmitted from viraemic to uninfected horses by *Cx. tritaeniorhynchus*. In parts of Japan where JEV was endemic, horses were attacked by thousands of *Cx. tritaeniorhynchus* daily (Gould *et al.*, 1964). Most non-porcine domesticated mammals are considered dead-end hosts. By providing blood they assist vector multiplication, but when serving as blood sources to infective mosquitoes they may dampen virus propagation.

(c) Bats

JEV has been isolated from a number of bat species, and their role as possible amplifying hosts has attracted interest. The order Chiroptera consists of two suborders. (i) Megachiroptera, which contains the single family Pteropodidae (fruit bats and flying foxes), of which the genera cited here are *Pteropus* and *Rousettus*. (ii) Microchiroptera, which includes, among other families, the Rhinolophidae (genera cited: *Hipposideros* and *Rhinolopus*), Vespertilionidae (genera cited: *Eptesicus*, *Miniopterus* and *Pipistrellus*), and Emballonuridae (genus cited *Taphozous*). Experimental studies have shown bats to be more susceptible to JEV when experimentally inoculated than when bitten by infected mosquitoes. The brief review that follows principally notes observations on natural transmission.

Relatively little is known of JEV infection in fruit bats and flying foxes. In India, the fruit bat *Cynopterus sphinx* developed viraemias after being bitten by infective *Culex bitaeniorhynchus*, and 21 days post-bite showed anti-JEV HI and neutralizing antibodies (Banerjee *et al.*, 1984a). In southern China, where the prevalence of JE is high, JEV-specific antibodies were detected in serum samples from *Rousettus leschenaultii*: 17.8% in samples tested by ELISA (n = 197) and 31.4% as neutralizing antibodies (n = 35) (Cui *et al.*, 2008). In laboratory experiments in Australia, of ten flying foxes (*Pteropus alecto*) exposed to the bites of JEV-infected *Cx. annulirostris* none developed clinical symptoms or detectable viraemias, but by 14 days post-bite six had formed JEV-specific IgG antibodies, and mosquitoes that fed on two of those bats became infected (van den Hurk *et al.*, 2009b).

More investigations have been undertaken on JEV infection in species of Microchiroptera. Screening blood samples from insectivorous bats on the Japanese islands of Honshu and Kyushu showed JEV to be present in *Miniopterus schreibersii* (16 isolates; n = 1139) and *Rhinolophus cornutus* (8 isolates; n = 267). Isolates were obtained with about equal frequency during each of four seasons (spring, summer, late autumn and winter), and JEV was present in brown adipose tissue in addition to blood. Among the microchiropteran species, of which over 20 specimens were tested for anti-JEV neutralizing antibody, seropositivity was found in eight species, with a mean prevalence rate of 8.5% (n = 1443) (Miura *et al.*, 1970; Sulkin *et al.*, 1970). In Taiwan, JEV was isolated from bats captured in caves: one isolate was from *Hipposideros armiger terasensis* (n = 3), and two were from *Miniopterus schreibersii* (n = 66); all three isolates were from samples collected on the same day and from a cave in which JEV-positive *Culex annulus* were present (Cross *et al.*, 1971). In southern China, anti-JEV antibodies were detected in serum samples from 10% of *Taphozous melanopogon* (n = 30), and 10.8% of *Miniopterus schreibersii fuliginosus* (n = 46) (Cui *et al.*, 2008). In southern India, anti-JEV neutralizing antibodies were present in *Rhinolophus rouxii* and in four species of *Hipposideros*; prevalence varied markedly from one year to the next (Banerjee *et al.*, 1988). In North America, individuals of *Eptesicus fuscus* became infected with JEV by the bite of infective *Culex pipiens*; 4 days later, *Cx. quinquefasciatus* that fed on those infected bats became infected. An individual *Pipistrellus subflavus* developed a viraemia after ingesting three mosquitoes infected with JEV. Mosquitoes have been found among the stomach contents of bats (La Motte, 1958).

To test whether bats might remain infected with JEV throughout a period of hibernation, ten individuals of *Eptesicus fuscus* (one of which later died) were inoculated with $10^{3.8}$ LD_{50} JEV and immediately placed at 10°C, simulating the temperature in their cave hibernacula in Maryland and Pennsylvania. After an interval of 36 days (bats 1–3), 77 days (bats 5–7) or 107 days (bats 8–10) were transferred to 24°C, to be bled immediately and again after 3, 6 and 9 days. On first return to room temperature no virus was detectable, but after 3 days viraemias occurred in all individuals, reaching a maximum at 3 or 6 days after transfer to 24°C, at titres infective to feeding mosquitoes (La Motte, 1958).

(d) Insectivores and rodents

The house shrew *Suncus murinus* (Soricidae, Insectivora) could be experimentally infected with JEV by mosquito bite, and mosquitoes could be infected by feeding on viraemic shrews (Banerjee *et al.*, 1984a).

Relatively little is known of JEV infections in rodents. In Japan, wild rodents were considered potential hosts of JEV because they were susceptible to infection, had large yearly population turnovers and were widely distributed. In Tokyo City, blood samples were taken from 270 trapped specimens of *Rattus* spp. (Muridae), and on the Kantō Plain blood samples were taken from 163 trapped specimens of seven species, mostly from two species of *Apodemus* (Muridae). Among the total of 433 rodents, only two specimens of *Apodemus speciosus* showed evidence of anti-JEV antibodies. Scherer *et al.* (1959e) concluded that wild rodents did not play an important role in the ecology of JEV in that region, and they surmised that the low infection rate reflected infrequent biting by *Cx. taeniorhynchus*.

After intracerebral inoculation into laboratory mice, JEV multiplies in the brain tissues. Significantly larger amounts of virus are produced in the brains of very young compared with adult mice. Viraemia develops, but virus titres are greater in the brain than in the blood. Sera from infected adult mice have been used as a source of HI and CF antibodies (Clarke and Casals, 1958).

(e) Primates

Serological tests have shown Japanese and South-east Asian monkeys to be naturally infected with JEV. Use of PRNT test blood samples from Japanese macaques (*Macaca fuscata*) in Japan, and long-tailed macaques (*M. fascicularis* (cited as *M. iris*)) imported to Japan from South-east Asia in 1963–1966 or 1978, produced the following seropositivities by country of origin: Japan, 29.7%; Cambodia, 9.0%; Vietnam, 8.6%; Indonesia, 2.7%; Philippines, 1.4%; Malaysia, 0% (Yuwono *et al.*, 1984). In the Philippines, tests by IgM capture ELISA showed 38.6% positivity among feral *M. fascicularis* aged 7–10 years (n = 44), leading Inoue *et al.* (2003) to surmise that a sylvatic cycle of JEV may exist.

Humans are dead-end hosts of JEV. The virus replicates in them but does not produce viraemias.

45.4.5 Mosquito hosts and vectors

From detailed evidence it was established that most putative vectors of JEV are species of the Sitiens Group of *Culex* (*Culex*). Of the species listed below, *Culex tritaeniorhynchus* and *Cx. gelidus* have satisfied the full range of tests needed to establish that they are vectors in natural transmission cycles; the other species are putative vectors.

Subfamily Culicinae
  Tribe Culicini
    Culex (*Culex*):
      Pipiens Group:
        Gelidus Subgroup: *Cx. gelidus*
        Univittatus Subgroup: *Cx. fuscocephala*
      Sitiens Group:
        Sitiens Subgroup: *Cx. annulirostris*, *Cx. palpalis*, *Cx. sitiens*
        Vishnui Subgroup: *Cx. annulus*, *Cx. pseudovishnui*, *Cx. tritaeniorhynchus*, *Cx. vishnui*
    Culex (*Oculeomyia*): *Cx. bitaeniorhynchus*
  Tribe Aedini:
    Armigeres: *Ar. subalbatus*.

Systematics: Sirivanakarn (1975, 1976); Chapman *et al.* (2000); Harbach (2011c).

Ground pools provide the larval habitat of most of these *Culex* species, and drought conditions that reduce large water bodies to small pools can produce an increase in adult population densities. The aquatic stages of many of these species thrive in irrigated rice fields. The adult females readily attack large mammals, including pigs, cattle and humans, and some feed on birds also. In any location, the distribution of blood feeds between the different host species is affected by their relative attractiveness, relative numbers and relative biomass.

The status of *Culex tritaeniorhynchus* as a natural host and vector of JEV in Japan and elsewhere has been established by the following lines of evidence. (i) JEV has been isolated from *Cx. tritaeniorhynchus* in the field. (ii) The flight range and flight periodicity of *Cx. tritaeniorhynchus* overlap with the distribution and temporal accessibility of avian and porcine hosts of the virus, and it feeds on those hosts. (iii) The seasonal period of host-feeding activity of *Cx. tritaeniorhynchus* coincides with that of disease incidence (Buescher and Scherer, 1959; Buescher *et al.*, 1959a,b; Scherer *et al.*, 1959a,c). (iv) Infection of *Cx. tritaeniorhynchus* followed ingestion of as little as $10^{1.9}$ mouse LD_{50} ml^{-1} pig blood, and these mosquitoes could transmit it to plumed or great egrets (*Ardea alba*) (Gresser *et al.*, 1958). (v) JEV replicated rapidly in *Cx. tritaeniorhynchus*, and virions accumulated in the salivary glands (Shichijo *et al.*, 1972; Takahashi and Suzuki, 1979). (vi) In the laboratory, *Cx. tritaeniorhynchus* transmitted JEV from infective to uninfected pigs or birds, even when viraemia titres were low (Gresser *et al.*, 1958). (vii) In the field, caged, uninfected birds and pigs became infected by the bites of wild *Cx. tritaeniorhynchus* (Buescher *et al.*, 1959a). (viii) *Culex tritaeniorhynchus* has an opportunistic host-feeding pattern, distributed between mammals and birds (Volume 2, Section 39.3.1.d).

Culex tritaeniorhynchus is the main vector of JEV over most of the virus's range (Table 45.11).This mosquito is widely distributed in the Old World. In longitude, its range extends from West Africa to Japan, the Philippines, Sulawesi and East Timor; in latitude, it extends from Japan and Korea to southern Africa. At one time, populations in the eastern part of the range were ranked as a subspecies, *Cx. t. summorosus*, but that distinction is no longer accepted as valid (Knight, 1978). Marshes provide larval habitats for *Cx. tritaeniorhynchus* over most of its geographical range, but irrigated rice fields are exploited wherever they are constructed. At certain stages in the cycle of planting and harvesting rice, conditions are suitable for oviposition and larval development. The adult females feed readily on large mammals, especially pigs, and to a lesser extent on birds (see Tables 45.13, 45.14, 45.15). Throughout much of its geographical range, *Cx. tritaeniorhynchus* obtains most blood meals from cattle, which do not produce infective viraemias (van den Hurk *et al.*, 2009a, review). In parts of India where cattle greatly outnumbered pigs, the effect of cattle as dead-end hosts was thought to

Table 45.11 Established and putative culicid vectors of Japanese encephalitis virus in different countries.

Country	*Species*	*References*
Japan, including the Ryuku Archipelago	*Culex tritaeniorhynchus*	1, 2
China (mainland)	*Cx. tritaeniorhynchus*	3
Taiwan	*Culex annulus*, *Cx. taeniorhynchus*, *Armigeres subalbatus*	4, 5, 6
North Thailand	*Cx. tritaeniorhynchus*, *Culex gelidus*, *Culex fuscocephala*	7, 8
South India	*Cx. tritaeniorhynchus*, *Culex vishnui*, *Cx. fuscocephala*, *Cx. gelidus*	9, 16
Sarawak State (Malaysia)	*Cx. tritaeniorhynchus*, *Cx. gelidus*	10
Java (Indonesia)	*Cx. gelidus*, *Cx. fuscocephala*, *Cx. tritaeniorhynchus*	11, 12
Papua New Guinea	Sitiens Subgroup of *Culex*	13
Torres Strait islands, Cape York Peninsula (Queensland, Australia)	*Culex annulirostris*, *Cx. gelidus*	14, 15

Culex tritaeniorhynchus and *Cx. gelidus* are proven natural vectors of JEV. Varieties of evidence support the identification of the other species as putative natural vectors.

The references are to papers that record local involvement of the named mosquitoes: 1, Buescher *et al.* (1959a); 2, Ura (1976a); 3, Huang (1982). 4, Wang *et al.* (1962); 5, Detels *et al.* (1970); 6, Chen *et al.* (2000); 7, Leake *et al.* (1986b); 8, Gajanana *et al.* (1997); 9, Gould *et al.* (1974); 10, Simpson *et al.* (1974); 11, Van Peenen *et al.* (1975a); 12, Van Peenen *et al.* (1975b); 13, Johansen *et al.* (2000); 14, Hanna *et al.* (1996);. 15, Hanna *et al.* (1999); 16, Arunachalam *et al.* (2009).

account for the lower seroconversion rates in children (Arunachalam *et al.*, 2005). Reisen *et al.* (1976) reviewed the effects of climate on the adult population densities of *Cx. tritaeniorhynchus*. In the temperate and subtropical zones, the annual curves for population density closely paralleled the temperature curves. As latitude increased through those zones, the season of adult activity became progressively shorter, producing a sharper peak of population density. In contrast, in the tropical zone, population densities were closely related to patterns of available water supply, whether in the form of rain, rice-field irrigation, or both.

Culex gelidus satisfies all the criteria needed to be proved a natural vector (Gould *et al.*, 1962; Simpson *et al.*, 1970b). It feeds readily on pigs, and in Malaysia maintains the mosquito–pig transmission cycle. It is a local vector on islands in the Torres Strait, having sufficient vectorial capacity to maintain localized endemicity in the absence of the main vector (Simpson *et al.*, 1970b; van den Hurk *et al.*, 2001).

Culex annulus is a putative vector in Taiwan, where it is far more abundant than *Cx. tritaeniorhynchus* and *Cx. fuscocephalus*. JEV has been isolated from females captured in pig-baited traps and from females caught in caves (Detels *et al.*, 1970; Cross *et al.*, 1971).

Culex annulirostris is a putative vector in Papua New Guinea, on islands in the Torres Strait, and in Cape York Peninsula (Queensland). JEV has been isolated from wild-caught females, and transmission was achieved experimentally with wild-caught females. This mosquito feeds preferentially on marsupials and opportunistically on other mammals and birds (van den Hurk *et al.*, 2003).

Culex vishnui is a putative main vector in South India and local vector in northern Thailand. It feeds primarily on mammals but attacks birds also. JEV has been isolated from wild-caught females, and transmission was achieved experimentally in the laboratory (Banerjee *et al.*, 1984b; Leake *et al.*, 1986b; Reuben *et al.*, 1988; Murty *et al.*, 2002).

Culex fuscocephala is a putative local vector in South India and Java, where JEV has been isolated from wild populations of the mosquito. It feeds primarily on mammals, including cattle and pigs (Van Peenen *et al.*, 1975a; Reuben *et al.*, 1992; Gajanana *et al.*, 1997).

Armigeres subalbatus is a putative local vector. JEV has been isolated from *Ar. subalbatus* in Japan (Fukumi *et al.*, 1975). Its larvae inhabit containers holding heavily polluted water. *Armigeres subalbatus* was thought to be a local vector on the small island of Liu-ch'iu, off Taiwan, where anti-JE antibodies had been detected in pigs and humans. The island was 'rice free', and none of the other three mosquito species was a putative vector. When JEV that had been isolated from *Ar. subalbatus* on Liu-ch'iu was fed from a suspension to colonized *Ar. subalbatus*, the virus disseminated to the salivary glands of 79% of females (Chen *et al.*, 2000).

45.4.6 Transmission cycles in Japan – basal studies

Research undertaken in Japan between 1950 and 1975 led to identification of the amplifying hosts of JEV and of its main vector, and established the nature of the enzootic and amplification cycles. Comparable research has not been undertaken in such detail elsewhere.

(a) Research locations and their faunas

The islands of Japan have a north-east to south-west alignment. The main islands are situated mostly within the temperate zone but extend into the subtropical zone (34° N – 23.5° N), while the Ryuku archipelago is entirely within the subtropical zone (Figure 45.20). The pattern of JEV transmission varies with latitude over this range. Early fieldwork (1952–1957) was undertaken at a number of locations on the Kantō Plain of Honshu, the largest of the main islands. In later years (1964–1973) these investigations were extended to the Nagasaki area and Taniyama on Kyushu Island, situated slightly to the south, and to two islands in the Ryuku archipelago (Table 45.12).

The Kantō Plain, which occupies an area of some 9000 km^2, represented the inhabited coastal regions of Japan. During the 1950s it had a population of at least 12 million people, including the inhabitants of Tokyo city, which is situated at

Table 45.12 The locations of investigations undertaken in Japan into the transmission of Japanese encephalitis virus that are mentioned in this chapter. The map omits the Ryuku Archipelago, a chain of small islands that extends for over 700 km south-eastwards from Kyushu Island.

Location	*Approximate latitudes*
Hokkaidō	
Sapporo city	43° 03' N
Honshu	
Miyagi Prefecture	37° 50'–39° 00' N
Kantō Plain	35° 50'–36° 20' N
Shinhama heronry	
Sagiyama heronry	
Daiganji cormorant colony	
Irumagun	
Zama	
Tokyo city	35° 35'–35° 45' N
Chiba	35° 35' N
Okayama city	34° 40' N
Kyushu	
Nagasaki area	32° 40'–33° 05' N
Taniyama	31° 30' N
Ryuku Archipelago	
Amami Islands	28° 00'–28° 31' N
Okinawa Islands	26° 27'–26° 52' N

the eastern edge of the plain. At the time of the first investigations, human epidemics of Japanese encephalitis occurred annually. The rural areas of the Kantō Plain contained extensive rice paddies, crops of barley or millet, fields of tea or vegetables, and many small piggeries. The arable parts of the plain were interspersed with large areas of mixed woodland and other more natural habitats. The region contained a number of large heronries, notably at Sagiyama (a National Preserve) and Shinhama, and a cormorant colony at Daiganjii. Azure-winged magpies (*Cyanopica cyanus*) were studied at Irumagun, an upland area of farmland interspersed with woodland. Two study sites in the Chiba region, north-east of Tokyo Bay, contained large breeding colonies of ardeids and a colony of cormorants (Scherer and Buescher, 1959).

During the period May to September each year, 2000–4000 birds of the family Ardeidae (Table 45.9) were present at Shinhama heronry, mostly black-crowned night herons, little egrets and intermediate egrets. About 200 little egrets remained during the winters. From April to October, about 3000–5000 ardeids were present at Sagiyama heronry – the three species named above, and also great egrets and cattle egrets. The annual appearance of ardeids at Shinhama and Sagiyama in the spring and their disappearance in the autumn were evidence of migration (Buescher *et al.*, 1959b; Scherer *et al.*, 1959b).

Pigs were present in farms throughout the Kantō Plain, and were overwhelmingly the most abundant domesticated animals: the estimated pig population within a 3-mile radius of Sagiyama was 500–1000. The farrowing of sows was completed by April, and pigs were slaughtered in large numbers in June or September. Other domesticated animals included oxen, cows, sheep, goats, dogs, ducks and chickens (Scherer *et al.*, 1959c).

(b) Analytical methods

In tests for the presence of antibodies in plasma samples, the Nakayama strain of JEV was used as

antigen and seed virus in neutralization (NT), complement-fixation (CF), and haemagglutination-inhibition (HI) tests (Buescher *et al.*, 1959c). Of these three assays, the HI test was the most frequently used because of its sensitivity and technical simplicity, in spite of its poor specificity (Buescher *et al.*, 1959b,d). The investigators were confident that the antibodies they detected were JEV specific because, at that time, Japan was 'free from other coexisting, immunologically related viruses, such as occur in the tropics' (Scherer and Buescher, 1959). Those were the so-called Group B viruses (flaviviruses), namely DENV, SLEV, WNV, YFV and JEV (cited as Japanese B virus) (Clarke and Casals, 1958; Westaway and Blok, 1997). In some later studies, the use over a period of time of a modified HI test involving 2-mercaptoethanol (Section 44.3.4.a) indicated the approximate date of first infection.

To test for the presence of JEV in mosquito or vertebrate hosts, mosquito extracts or blood samples were inoculated intracerebrally into infant mice, and antigens extracted from brain tissue of the mice that became sick were examined further (Buescher *et al.*, 1959a,b).

Among birds, the nestlings and flightless juveniles of ardeids were screened in large numbers; cormorants, azure-winged magpies and other birds were also screened. Only small numbers of adult birds were collected by shooting (Buescher *et al.*, 1959b; Scherer *et al.*, 1959b). Blood samples were taken for screening from pigs and other domesticated animals, and from children aged 6–12 years living near the Shinhama and Sagiyama study areas (Scherer *et al.*, 1959c,d).

(c) Viral infections in birds

Most studies in Japan have been on species of Ardeidae (herons, egrets, bitterns). Over a number of years, approximately 5% of the ardeid populations at the Shinhama and Sagiyuma heronries were tested annually for JEV infection. For practicability, the study was confined to nestlings and juveniles. The demonstration of viraemia in a number of species raised the possibility of their being amplifying hosts.

During the summers of 1952–1956, nestlings and unfledged juveniles of black-crowned night herons, intermediate egrets and little egrets were regularly but inapparently infected with JEV. The virus could be isolated from viraemic birds only during a period of 2–4 weeks in late summer. The prevalence of infection among ardeid nestlings tended to rise and fall during the period of virus dispersion, and varied annually from 5% to 50%. Prevalence was always greater in black-crowned night herons than in intermediate or little egrets, possibly reflecting the greater responsiveness of *Cx. tritaeniorhynchus* to the herons. Uninfected adult ardeids placed in baited or sentinel traps at ground level became infected with JEV, as did a high percentage of uninfected black-crowned night herons in traps placed in the tree tops (Buescher *et al.*, 1959b; Scherer *et al.*, 1959b).

JEV appeared to be carried over distances of 25–30 km from rural foci to areas of high human population density, such as Tokyo. It was thought unlikely that this could be explained solely by the flight of infected mosquitoes. Adult ardeids flew up to 30 km or more to obtain food, and Scherer *et al.* (1959b) surmised that JEV was dispersed through the daily movements of viraemic birds. These flights, it was suggested, could initiate secondary infection cycles among pigs and birds near cities, leading to the infection there of thousands of *Cx. tritaeniorhynchus*.

In the Kantō Plain investigations, few birds other than the ardeids were examined. In a study of azure-winged magpies, neutralizing antibodies to JEV were found only twice during the period 1953–1956, and the azure-winged magpies at Irumagun were thought not to play an important role in the dissemination of JEV (Buescher *et al.*, 1959b). Japanese cormorants produced their broods by early April, well before the seasonal dispersion of JEV. The presence of maternal antibodies to JEV in some cormorant nestlings indicated an earlier infection of the parent birds (Buescher *et al.*, 1959e).

(d) Viral infections in mammals

Before the 1950s, there had been little interest in the role of farmed pigs in the ecology of JEV. Blood

analyses revealed antibodies of maternal origin in piglets aged 2–7 months, but detectable antibodies had disappeared from most by 4–6 months of age, when they became susceptible to the virus. As these pigs grew, almost all became infected with JEV. Pigs used as bait in mosquito traps developed viraemias after becoming infected. Viraemias developed 1–3 days after inoculation with JEV, and lasted for 4 days. Apart from horses, which were dead-end hosts, no other domesticated mammals were infected to a significant degree (Scherer *et al.*, 1959c,g).

Severe epidemics of JE in humans occurred in Japan in 1924, 1935 and 1948. During the period 1948–1956, epidemics of varying size occurred near Tokyo each summer. Antibodies to JEV were found in individuals showing no clinical signs of infection as well as in patients with JE (Buescher *et al.*, 1959c; Scherer *et al.*, 1959d,f). Owing to preventive measures, there have been no epidemics in Japan since 1968, but sporadic cases still occur.

(e) Mosquito hosts and vectors

Mosquitoes were investigated at the named heronries, in rural locations, and in urban locations including the Setagaya district of Tokyo. They were captured in animal-baited traps set at ground level or at higher elevations, the bait including bird species of seven genera, pigs and humans. Because the size and surface area of hosts possibly affected mosquito responsiveness, the number of animals used in each trap was arranged to provide similar total body surface areas for comparisons of bird species, or for comparing the largest birds, pigs and humans (Scherer *et al.*, 1959a). Mosquito species attracted in large numbers to baited traps, or to resting stations occupied by cows or chickens, were tested for JEV (Buescher *et al.*, 1959a). The species captured in baited traps during these investigations included *Culex bitaeniorhynchus*, *Cx. pipiens*, *Cx. tritaeniorhynchus*, *Lutzia halifaxii*, *Anopheles sinensis*, *Stegomyia albopicta*, *Aedimorphus vexans nipponii* and *Armigeres subalbatus*. Only *Cx. tritaeniorhynchus*, *Cx. pipiens* and *An. sinensis* were caught in substantial numbers, and *An. sinensis* was caught only in pig-baited traps in rural areas (Scherer *et al.*, 1959a).

When traps baited with bird species from seven genera were placed at two locations, *Cx. tritaeniorhynchus* responded most strongly to black-crowned night herons, much less strongly to little egrets and (domesticated) chicks, and hardly at all to four other species (Table 45.13). In contrast, chick-baited traps equalled or bettered traps baited with black-crowned night herons in capturing *Cx. pipiens*. Small numbers of *Cx. tritaeniorhynchus* were

Table 45.13 Mosquitoes captured in small traps, placed at ground level and baited with birds, at Shinhama heronry and the nearby village of Minami-gyotoku on the Kantō Plain, Honshu, Japan, May–August 1953. (From the data of Scherer *et al.*, 1959a.) The number of birds per trap was varied with species to give comparable total areas of body surface.

Mosquito/Location	*Captures in traps baited with*							*Total numbers captured*
	Heron (%)	*Egret* (%)	*Chick* (%)	*Magpie* (%)	*Sparrow* (%)	*Thrush* (%)	*Starling* (%)	
Culex tritaeniorhynchus								
Shinhama heronry	87.6	5.6	6.8	0.02	0.02	0	0	4,761
Minami-gyotoku	66.9	21.5	7.7	1.1	1.5	0.8	0.4	1,893
Culex pipiens								
Shinhama heronry	41.0	10.1	37.3	4.0	4.2	0.4	3.0	1,152
Minami-gyotoku	23.3	14.4	31.3	7.3	5.2	7.1	11.4	37,332

Number of birds per trap: black-crowned night heron, 2; little egret, 2; domesticated chick, 3 or 4; azure-winged magpie, 3; Eurasian tree sparrow, 8; dusky thrush, 3; grey starling, 3 or 4.
See Table 45.9 for Latin names of bird species.

captured in bird-baited traps suspended at 7.3–15.2 m in the canopy. To assess the relative responsiveness of mosquitoes to birds, pigs and humans, appropriately baited traps were exposed together at three locations (Table 45.14). Under these competitive conditions, *Cx. tritaeniorhynchus* responded overwhelmingly to pigs. *Culex pipiens* responded most strongly to birds, but appreciably to pigs (Scherer *et al.*, 1959a).

Culex tritaeniorhynchus was the main vector of JEV in Japan, and no other species was an important subsidiary vector. In one survey, mosquitoes that were captured in bird-, pig- or human-baited traps, or collected from resting stations occupied by cows or chickens, yielded 300 isolates of JEV, of which *Cx. tritaeniorhynchus* yielded 298 and *Cx. pipiens* only two. No JEV was isolated from *An. sinensis* (Buescher *et al.*, 1959a).

In the cool-temperate region of Japan, *Cx. tritaeniorhynchus* is bivoltine. One generation produces adults that emerge in late summer or autumn, the females mating and then entering hibernation without blood feeding. They emerge from hibernation in early spring, blood feed and lay eggs. The second generation has a spring–summer lifespan. The mosquito vectors of JEV must fly from the rice-fields that are their larval habitats to the villages where they feed, and back to rice fields for oviposition. In mark–recapture experiments undertaken over 7 days with wild-caught female *Cx. tritaeniorhynchus* in a hilly area near Nagasaki, the mosquitoes dispersed along valleys and the sea coast. The mean distance travelled during the 7 days was 1.0 km. However, the mean distance flown on the first day was 0.9 km and the maximum 5.1 km, while during the 7-day period the maximum was 8.4 km. Some females travelled at least 2.0 km in a single flight (Wada *et al.*, 1969).

45.4.7 Seasonality of transmission in Japan

The research findings described in the previous section suggest that JEV exists in two transmission cycles: (i) an enzootic cycle involving ardeids and *Cx. tritaeniorhynchus*; and (ii) an amplification cycle involving the same mosquito and farmed pigs. *Culex tritaeniorhynchus* is also the vector involved in transmission to humans and horses – both dead-end hosts (Figure 45.21). In their study of the

Table 45.14 Numbers of mosquitoes captured in Magoon ground traps baited with pigs, birds (black-crowned night herons) or a human at three locations on the Kantō Plain, Honshu, Japan, June – September 1956. (From the data of Scherer *et al.*, 1959a.)

Mosquito	*Location*	*Captures in baited traps*			*Numbers captured*
		Pigs (%)	*Birds (%)*	*Human (%)*	
Culex tritaeniorhynchus	Sagiyama	95.2	4.6	0.2	126,450
	Zama	96.9	2.6	0.4	4,251
	Tokyo	81.8	14.3	3.8	286
Culex pipiens	Sagiyama	42.2	46.6	11.2	412
	Zama	28.6	62.7	8.6	706
	Tokyo	11.0	76.0	13.0	830
Anopheles sinensis	Sagiyama	98.0	0.3	1.6	15,025
	Zama	98.8	0.3	0.9	573
	Tokyo	0	0	0	0

Locations: Sagiyama heronry; Zama, a wooded area amidst rice fields, ecologically similar to Sagiyama; Tokyo, Setagaya district, densely populated. Magoon traps were baited with two pigs aged 3–6 months, or 12 herons, or one man.

epidemiology of Japanese encephalitis in the Kantō Plain, Buescher and Scherer (1959) divided the annual manifestation of JEV into three phases, here renamed: (i) the pre-appearance phase; (ii) the dispersion phase; and (iii) the disappearance phase. The same three seasonal phases are used here in descriptions of JEV seasonality at different latitudes in Japan: in the Kantō Plain, near Tokyo city, in the Nagasaki area and on two island groups in the Ryuku Archipelago – Amami and Okinawa (Table 45.12). But, first, the photoperiodism and overwintering of the main vector are examined.

(a) Photoperiodism and overwintering of Culex tritaeniorhynchus

In the Nagasaki area, exposure of *Culex tritaeniorhynchus* pupae to the short day lengths typical of late summer induced the adult females to enter diapause. During early dormancy, the primary ovarian follicles of these females were undeveloped (in Christophers' stages G to Ia). The females did not take blood meals until the completion of diapause (Kawai, 1969; Oda and Wada, 1973; Oda *et al.*, 1978).

At locations where hibernating females of *Cx. pipiens* and *An. sinensis* were easily found, hibernating females of *Cx. tritaeniorhynchus* were only very seldom found despite many attempts. On the Kantō Plain, ten female *Cx. tritaeniorhynchus* were found during the 1957–1958 winter in brush and wood piles and in a cave. They were unengorged, showed no ovarian development, and had fat-body stores that declined in amount with capture date (Bullock *et al.*, 1959). In late January 1967, 60 females of *Cx. tritaeniorhynchus* and 32 of *Cx. pipiens pallens* were found in caves in Chiba Prefecture and on the nearby Izu Peninsula. The *Cx. tritaeniorhynchus* were nulliparous, and tests for JEV proved negative (Wada *et al.*, 1968).

The finding, in the Nagasaki area, of a female *Cx. pipiens pallens* hibernating in a small cavity in a bank of earth encouraged the search for *Cx. tritaeniorhynchus* in similar situations. Mosquito netting was placed over the sides of earth banks or stone walls between fields, and the traps were examined after dark. Between 18 March and 30 April 1965, nine female *Cx. tritaeniorhynchus* emerged into the netting traps, of which one, caught on 27 April, had engorged. CO_2 traps placed in the fields first caught females at the end of March, and the catch size increased substantially with date through April. All those females were unengorged (Table 45.15). The first captures of *Cx. tritaeniorhynchus* in animal houses came 9 days after the first catches in CO_2 traps (Omori *et al.*, 1965). The mosquitoes caught in netting traps placed over banks and walls were, almost certainly, emerging from hibernation sites, and the investigators considered that those captured by other means

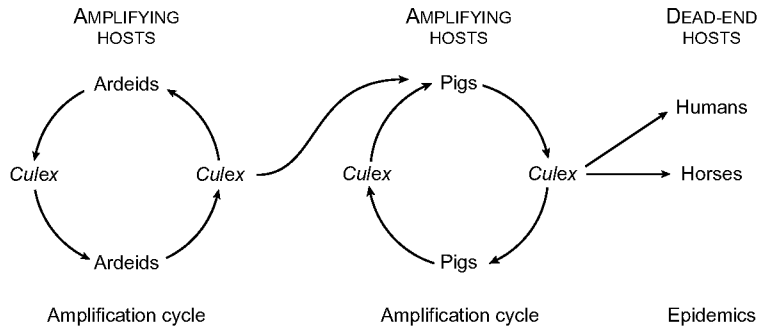


Figure 45.21 Transmission cycles of Japanese encephalitis virus in Japan during the years of high Japanese encephalitis prevalence. The mosquito hosts and vectors are *Culex tritaeniorhynchus*. The known amplifying hosts are water birds of the family Ardeidae and farmed pigs. Dead-end hosts are humans and horses. The arrows indicate the direction of virus transmission.

Table 45.15 First captures of *Culex tritaeniorhynchus* by trapping in nine villages in the Nagasaki area of Kyushu, Japan, in spring 1965. (From the data of Omori *et al.*, 1965.) The selected villages had terraced rice fields or livestock. Mosquitoes were captured from under netting placed on the sides of grassy banks or stone walls, in CO_2 traps placed in terraced fields, from animal houses, and from light traps placed near houses. All the *Cx. tritaeniorhynchus* captured were female. The total number captured was 20,291.

	Banks, walls		*CO_2 traps*		*Cowsheds*		*Pigsties*		*Henhouses*		*Light traps*		*Mean temp.*
Date	*Unfed*	*Fed*	*Unfed*	*Fed*	*Unfed*	*Fed*	*Unfed*	*Fed*	*Unfed*	*Fed*	*Unfed*	*Fed*	*(°C)*
March													
16–23	1	0	0	0	0	0	0	0	0	0	0	0	5.8–12.0
24–31	0	0	4	0	0	0	0	0	0	0	0	0	6.9–12.9
April													
1–7	0	0	25	0	0	0	0	0	0	0	0	0	8.7–13.6
8–14	3	0	424	0	27	61	11	11	8	0	22	10	10.6–14.8
15–21	0	0	1,749	0	409	341	55	74	55	2	53	552	12.3–15.3
22–28	4	1	8,792	0	185	519	1,318	2,054	115	41	406	1,702	15.2–19.9
29–30	0	0	133	0	96	370	81	461	38	63	7	8	11.6–12.9
Total	8	1	11,127	0	717	1,291	1,465	2,600	216	106	488	2,272	

during early and mid-April had also hibernated locally. As discussed in the following subsection, this interpretation was contested by Min and Xue (1996).

On the Amami Islands of the Ryuku Archipelago, which are situated well within the subtropical zone, the 'winters' are mild. At Naze on Amami-Oshima (28° 21' N), during December–January 1971–1974, the 10-day average temperature ranged between *c.* 12°C and 17.5°C. Females of *Cx. tritaeniorhynchus* that emerged in autumn entered a 'weak diapause', but acquired the ability to blood feed soon afterwards, and were observed blood feeding on some warm winter days (Wada *et al.*, 1976). Slightly further south, on Okinawa, the depth of diapause was lower still, and blood feeding and gonotrophic activity continued at a low rate, even during cooler winters (Iha, 1971). In southern Taiwan, at about 22° 30' N, *Cx. tritaeniorhynchus* could be found throughout the year, but the numbers were much diminished during the winter months (Hu and Grayston, 1962).

(b) Pre-appearance phase

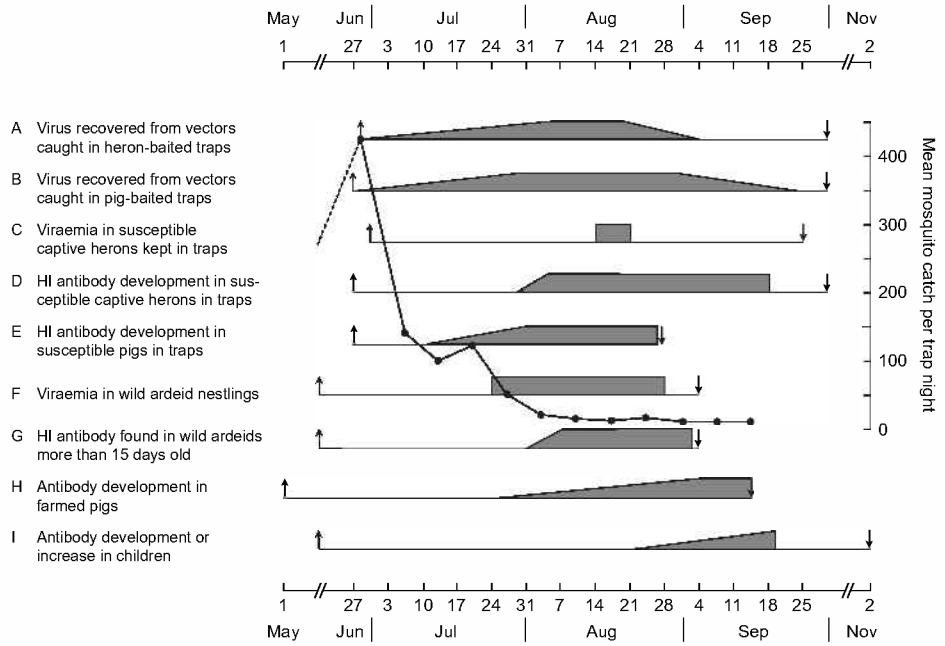
This section concerns the first appearances in spring of ardeid hosts, *Culex tritaeniorhynchus* and JEV.

The **Kantō Plain** is an area of 16,172 km^2 of lowland facing the Pacific Ocean, and is the most populous area of the country. It is walled by mountains to the north and west and by hills to the south. JEV could not be isolated from pigs during the winter. By agricultural practice, most pigs were born during the periods September–November or February–April. In April, domestic sows completed farrowing (giving birth). Migratory ardeids arrived in the early spring from their tropical or subtropical overwintering sites, nested and started to produce young. No evidence of viraemia or of recent infections was obtained from birds sampled at that time (Figure 45.22). Hibernating *Cx. tritaeniorhynchus* could not be caught for examination because their hibernation sites were unknown. Adult females started to appear during April, and none were infected. Between April and late June, the aquatic stages of *Cx. tritaeniorhynchus* developed to produce adults of the first post-winter generation. At several sites near Tokyo, repeated investigation produced no evidence of infection in *Cx. tritaeniorhynchus* during the spring (April to June). JEV first became evident in *Cx. tritaeniorhynchus* in late June or early July, but its source was unknown. Hibernating female mosquitoes could not be eliminated as a possible source because only very few had been found. At Sagiyama in 1956, infected *Cx. tritaeniorhynchus* were found from late June onwards, well before infection of birds in the same habitat (Figure 45.22) (Scherer *et al.*, 1959a).

In the **Nagasaki** area, *Cx. tritaeniorhynchus* emerged during late March and April from hibernation sites in earth banks and stone walls, and at about the same time they could be captured in CO_2 traps (Table 45.15). Between 31 March and 30 April 1965, 11,127 female *Cx. tritaeniorhynchus*, none of which had blood fed, were caught in CO_2 traps in fields. In contrast, of 6395 females captured in animal houses during that period, 62.5% had blood fed (Omori *et al.*, 1965). During the period 1965 to 1973, the dates of first appearance of female *Cx. tritaeniorhynchus* in the Nagasaki Prefecture ranged from 13 March to 8 April. On average, males of the spring generation were first trapped 41 days after females had first emerged from hibernation (range 29–46 days) (Table 45.16). The investigators concluded that unfed females emerged from diapause between late March and April, and gave rise to a generation that emerged between late April and mid-May (Wada *et al.*, 1975).

Mosquitoes are among the insects that disperse over huge distances at high altitude, being carried in moving air masses. At a location in China, of 44 mosquitoes captured at 150–250 m above ground level and being displaced in a south-west direction, 31 were female *Cx. tritaeniorhynchus*. It was estimated that almost 2 million individuals per night were crossing each kilometre of an imaginary line on the ground (Ming *et al.*, 1993). Similarly, *Cx. tritaeniorhynchus* were trapped at 150 m above ground level in north-east India,

Figure 45.22 The sequence of infection of natural hosts (mosquitoes, ardeid birds, pigs, humans) with Japanese encephalitis virus in the Sagiyama study area on the Kantō Plain in Honshu, Japan, in 1956. (After Buescher and Scherer, 1959.) The superimposed curve shows the mean catch per trap night of *Culex tritaeniorhynchus* in heron-baited traps placed at ground level. (After Buescher *et al.*, 1959a.)



The Sagiyama study area included the Sagiyama heronry, which was situated on a long, raised crest of land about 32 km north of Tokyo. Immediately surrounding the heronry were dry fields of tea and vegetables, but within 0.8 km distance, and at 30 m lower elevation, were extensive rice paddies where *Cx. tritaeniorhynchus* developed. The heronry, which contained some 3000–5000 ardeids during the period April–September, had existed for hundreds of years and was a 'National Preserve'. Black-crowned night herons, plumed egrets and little egrets were present from April to September; great egrets and cattle egrets were found during June and early July. Most nests were at 6 m or above ground level. Humans, with their pigs and chickens, resided within the colony. Within a radius of 3 miles (4.8 km) around the heronry there was an estimated population of 500–1000 pigs, and the human density was 3000 mile^{-2} (2.59 km^{-2}) (Scherer and Buescher, 1959).

↑, Screening starts. ↓, Screening ends. Records are marked A to I, top to bottom. Records A and B provide direct evidence of mosquito infection. Records C–E, which are of infection in sentinel animals kept in traps, provide indirect evidence of infection in mosquitoes that entered the traps but are not relevant to times of infection in wild vertebrate hosts. Records F–I provide direct or indirect evidence of infections in wild birds, farmed pigs and humans. Catches of *Cx. tritaeniorhynchus* in pig-baited traps were much greater than those in heron-baited traps (curve), but showed a similar seasonal decline.
HI antibody, haemagglutination inhibition antibody.

variably travelling in a NE, NW or SSW direction (Reynolds *et al.*, 1996). Six female *Cx. tritaeniorhynchus* were among a total of 30 mosquitoes captured in light traps set up on a ship sailing between the south of Kyushu Island and Amami Island, the traps being open while the ship was 50 km distant from each port (Hayashi *et al.*, 1975). (See also Volume 2, Section 33.9.3.)

In a review of studies on the overwintering of *Cx. tritaeniorhynchus*, Min and Xue (1996) considered that the few females that had been found in hibernation sites, or that had clearly emerged from hibernation sites, could not

Table 45.16 For the period 1965–1973, the range of dates of first occurrence of four key events in the transmission of Japanese encephalitis virus in Nagasaki Prefecture. (From the data of Fukumi *et al.*, 1975; and Wada *et al.*, 1975.)

First appearances of:		*Range of dates*
i	Overwintered females	13 Mar–8 Apr
ii	Males of spring generation	25 Apr–15 May
iii	JEV in mosquitoes	30 May–9 Sep
iv	HI antibody in pigs	22 Jun–5 Sep

(i) Emergence of *Culex tritaeniorhynchus* from hibernation.
(ii) Emergence of adults of the following generation.
(iii) First isolation of JEV from *Cx. tritaeniorhynchus*.
(iv) First evidence of infection of pigs.
The number of days between first isolation of JEV from mosquitoes and first appearance of 2-ME sensitive haemagglutination antibody in farmed pigs in each year from 1965 to 1973 was 23, 10, –1, –2, –8, 20, –7, 20 and 14, respectively.

represent overwintering populations large enough to account for the size of the following generation. They had examined meteorological data for the dates of first appearance of *Cx. tritaeniorhynchus* in early spring, as reported by Japanese authors for 6 years between 1966 and 1971, and found that all coincided with the dates of passage of weather fronts. In China, continuous observations during the period 1982–1988 at the coast of Fengxian 'County', near Shanghai, showed that all early spring appearances of *Cx. tritaeniorhynchus* were associated with spells of wind from the south-west and occurred on the day when the front passed through, or on the following day, or in the sinking airflow in the tail of the high-pressure cell (Min *et al.*, 1989). Min and Xue (1996) concluded that in the cool-temperate region too few diapausing female *Cx. tritaeniorhynchus* survive the winter to produce the large following generation and that, rather, it is produced by wind-borne migrants from the south.

One piece of evidence from Japan is inconsistent with that view – a total absence of males from the *Cx. tritaeniorhynchus* captured in the Nagasaki area during March and April, e.g. from 2760 captured in light traps (Table 45.15). Some reports of wind-assisted movement of *Cx. tritaeniorhynchus* have recorded small numbers of males. Thus, of 91 *Cx. tritaeniorhynchus* caught in traps on a ship 200 km from land in the East China Sea, one was male and 90 female (Asahina, 1970), while, of 19 trapped 150 m above ground in West Bengal, four were male (Reynolds *et al.*, 1996). More information is needed to decide between the opposed viewpoints – whether in cool-temperate regions of Asia most of the spring and early summer generation of *Cx. tritaeniorhynchus* is produced by overwintered females or by wind-assisted migrant females. The possibly wind-assisted movement of *Cx. annulirostris* (another vector of JEV) from Papua New Guinea to the Cape York Peninsula is discussed in Section 45.4.2.c.

From 1964 to 1973, investigations were conducted annually in villages around Nagasaki and in the suburbs of Taniyama, which is also on the island of Kyushu. Attempts to isolate JEV from many females of *Cx. tritaeniorhynchus* thought to have emerged from hibernation gave negative results. During this period, the two earliest dates of capture of JEV-infected *Cx. tritaeniorhynchus* were 30 May and 8 June, from females of the post-diapause generation and shortly before the earliest recorded dates from the Kanto¯ Plain. In most years, the first isolation occurred later in June or in July (Table 45.17). The mosquito hosts of 232 isolates of JEV obtained during 1964–1973 in the Nagasaki area were as follows: *Cx. tritaeniorhynchus*, 93.5%; *Cx. pseudovishnui*, 3.4%; *Am. vexans nipponii*, 1.7%; *Cx. pipiens pallens*, 0.9%; and *Ar. subalbatus*, 0.4%. Only *Cx. tritaeniorhynchus* was thought to play an important role in the dissemination of JEV (Hayashi *et al.*, 1966, 1976; Fukumi *et al.*, 1975).

On the **Amami Islands** (28° 0' to 28° 31' N) in the Ryuku archipelago, females of *Cx. tritaeniorhynchus* that emerged in autumn acquired the ability to blood feed soon afterwards, and were observed blood feeding on some warm winter days. The finding of parous females during the winter indicated that ovarian development and oviposition had occurred. Larvae and pupae of the following generation were collected in March, and the adults emerged at the end of March. Females could be captured throughout the year in animal

Table 45.17 Monthly totals of Japanese encephalitis virus isolates from female *Culex tritaeniorhynchus* captured in villages in the Nagasaki area of Kyushu, Japan during the period 1964 to 1973. (From the data of Hayashi *et al.*, 1966; and Fukumi *et al.*, 1975.)

Month	*Isolates*	n
March	0	3,293
April	0	60,650
May	1 *	35,289
June	35	47,549
July	135	86,139
August	44	52,849
September	2 †	13,020
October	0	428
Totals	217	299,217

*, 30 May 1965. †, data from the period 1–6 September 1964–1973.

and poultry houses (Hayashi *et al.*, 1975, 1976; Wada *et al.*, 1976).

On Amami-Oshima Island, mosquitoes trapped in animal houses during February to November 1973 were screened for JEV. The virus was isolated from females captured during early and mid-February (4/8 pools, *n* = 1083), but no further isolates were obtained (0/26 pools, *n* = 1145) until mid-May. In 1974, no isolates of JEV were obtained from January to June (0/60 pools, *n* = 6536), and the first isolate was obtained in early July. In 1975, the first isolate was obtained in early June (Hayashi *et al.*, 1975). Owing to the low population density of *Cx. tritaeniorhynchus* in summer, a significant number of pigs remained susceptible to JEV in autumn. Wada *et al.* (1976) surmised that during warm winters JEV persists on Amami-Oshima Island through continuation of the pig–mosquito cycle, but that in other years JEV is reintroduced from elsewhere to initiate a summer cycle in pigs.

Further south, on **Okinawa Island** (26° 04' to 26° 51' N), blood feeding and gonotrophic activity continued throughout the relatively warm winter of 1968–1969, and even during cooler winters those activities continued at a low rate (Iha, 1971). Over a number of years, screening for JEV was undertaken weekly or biweekly by testing *Cx. tritaeniorhynchus* caught in dry-ice traps set up in cowsheds and pigsties, and also by testing pig sera. During the period 1966–1976, the first isolations of JEV from *Cx tritaeniorhynchus* usually occurred in early April to May. However, in 1976 at Ogimi village (c. 26° 35' N), JEV was isolated during the period 13–15 February (2/9 pools, *n* = 876), before emergence of the spring generation of these mosquitoes. In contrast, from pig sera the earliest isolation was on 25 May, at Nago village (c. 26° 42' N). This was taken as evidence of JEV overwintering in mosquitoes (Ura, 1976a,b). In 1977, a special effort was made at Ogimi village to capture overwintering mosquitoes with light and CO_2 traps for screening purposes, but no isolates of JEV were obtained during the period 20 January to 1 March (0/42 pools, *n* = 1935) (Ura, 1977).

Where records were available for 5 of the 6 years from 1969 to 1974, JEV was isolated from *Cx. tritaeniorhynchus* some 34–77 days (mean 57 days) earlier on Okinawa than in the Nagasaki area. Similarly, between 1966 and 1974, 2-ME-sensitive HI antibody appeared in pigs 51–84 days earlier on Okinawa than at Nagasaki (mean 67 days) (date ranges: Okinawa, 11 April–27 June; Nagasaki, 22 June–5 September). In some years on Okinawa, new infections appeared in pigs first, in other years in mosquitoes first (Hayashi *et al.*, 1976). Using additional data sets, Ura (1976a) reported similar but not identical findings.

(c) Dispersion phase

This section concerns transmission of JEV to its various hosts at different latitudes, and its geographical dispersion.

On the **Kantō Plain**, JEV infections in the vector and in its avian, porcine and dead-end hosts were mid- and late-summer phenomena. The populations of *Cx. tritaeniorhynchus* reached their maximum numbers by late June to early July, and shortly after this JEV was first detected in them. One month later the prevalence of infections in the vector populations reached its peak. From July until late September the vector populations declined, but this did not prevent transmission to

the many susceptible ardeids and pigs. Shortly after JEV had appeared in the mosquitoes, it was efficiently transmitted to birds and pigs, and thereafter in both directions. The prevalence of infections in the amplifying hosts increased during late July and August, but this increase came several weeks after that in the vectors. Pigs born during the spring had lost their maternal immunity by mid- or late summer, when they became susceptible to infection. The infrequent occurrences of encephalitis in horses were concurrent with the infections in pigs. Humans were not infected until August, when the density of infected vectors was maximum (Buescher and Scherer, 1959).

For a more detailed description of the temporal pattern of JEV infections in the Kantō Plain, reference is made to records from 1954–1956 of infection rates in mosquitoes, ardeids, pigs and schoolchildren in the **Sagiyama** study area. From year to year the periods of infection varied in duration, and emphasis is given to the findings from 1956 (Figure 45.22). The study area was a rural but densely populated area around the Sagiyama heronry. Among **mosquitoes**, *Cx. tritaeniorhynchus* infected with JEV could be found from late June to late September (Figure 45.22A,B). The adult *Cx. tritaeniorhynchus* population peaked in late June (shown by the curve superimposed on Figure 45.22), just before the onset of its infection with JEV, and then declined sharply. The number of infected mosquitoes increased during July and then remained high, with infection rates of 5–50% in late August (Buescher *et al.*, 1959a).

Among **ardeid** nestlings (of black-crowned night herons, intermediate egrets and little egrets) infection with JEV was a regular mid- to late-summer phenomenon. Its prevalence was greater in the black-crowned night herons than in the egrets. The onset of infection could be timed by the sudden appearance of viraemia in nestlings, and viraemic juveniles were detected for 2 to 4 weeks thereafter – overall, between 25 July and 6 September (Figure 45.22 F). The viraemias found in 12–13-month-old sentinel herons showed that such older birds could be infected naturally (Buescher *et al.*, 1959b). Among the ardeid nestlings <15 days of age, 30% of black-crowned night heron and intermediate egret nestlings possessed neutralizing antibody to JEV, whereas <10% of little egret nestlings did (Buescher *et al.*, 1959e). Great egrets and cattle egrets reared their young earlier, during May, June and early July, and by the time that JEV was present in wild mosquitoes no nestlings of those species were available (Scherer *et al.*, 1959b). An autumn dispersion of ardeids from the heronries to other locations on the Kantō Plain, coupled with the 1–2-week extrinsic incubation period in mosquitoes, may help to explain the continued occurrence of human disease into early October. Except for cormorants in 1953 and 1955, attempts to implicate non-ardeid birds in JEV transmission cycles were unsuccessful (Scherer *et al.*, 1959b,d).

Pigs born during the period February–April were 4 months or more old by August, when most were without maternal antibodies. Between late July and early September 1956, out of 98 pigs tested on farms near Sagiyama, 97 developed HI antibody to JEV (Figure 45.22 H) (Scherer *et al.*, 1959c). During 1956, blood samples were taken from 242 **schoolchildren** aged 6–8 years. Before the Japanese encephalitis season, 27% possessed detectable neutralizing antibody to JEV, but none showed clinical signs of encephalitis. During the period 21 August to 19 September, 5% of the schoolchildren developed HI antibody, having inapparent infections (Figure 45.22 I) (Scherer *et al.*, 1959d,g). In the Shinhama study area, the temporal patterns of infection were broadly similar (Buescher *et al.*, 1959a,b; Scherer *et al.*, 1959d). At this time, epidemics of JE occurred annually in **Tokyo city**. Buescher and Scherer (1959) thought that dispersion of JEV from rural foci over distances of 15–20 miles (24–32 km) was not easily explainable by the flight of infected mosquitoes, and they suggested that the daily feeding flights of viraemic birds were a more likely explanation. Once secondary foci had arisen in the small piggeries, a relatively small number of viraemic pigs could serve as sources of infection for thousands of *Cx. tritaeniorhynchus* – sufficient for transmission to humans.

The **Miyagi Prefecture** is situated further north on Honshu Island. During the summer of 1964, pigs at four abattoirs situated in four geographically different areas of the Prefecture, were sampled weekly. Results from HI assays revealed two phases of infection separated by 18 days (Figure 45.23). During the middle of July, about 20% of pigs abruptly showed anti-JEV HI antibody. The vectors responsible for this outbreak of JE were not identified. A second abrupt increase in prevalence of HI antibody to 100% occurred at the beginning of August, at which time also the isolation rate of JEV from the *Cx. tritaeniorhynchus* population reached maximum. A main outbreak of JE in humans occurred towards the end of August, followed after about 18 days by a minor outbreak (Konno *et al.*, 1966).

Further south on Honshu Island, in Okayama city, blood samples from juveniles (thought to be >30 days old) and adults of black-crowned night herons and intermediate egrets captured during August and September revealed HI antibody to JEV, some being 2-ME sensitive. At that time, Japanese encephalitis was prevalent among humans (Ogata *et al.*, 1970).

In the **Nagasaki** area of Kyushu Island, during the period 1964–1973, JEV was first isolated from spring-emerged, female *Cx. tritaeniorhynchus* an average of 48 days after the appearance of that generation (range 15–63 days) (Table 45.16). The two earliest dates of capture of JEV-infected *Cx. tritaeniorhynchus* were 30 May and 8 June. The annual period of JEV dispersion among mosquitoes lasted between 15 and 100 days (mean 38 days). The number of isolations peaked in July, and by early September was very low (Table 45.17). JEV dispersed within the *Cx. tritaeniorhynchus* population before or during the peak of population density (not, as on the Kantō Plain, after the peak). The relative dates of first appearance of 2-ME-sensitive HI antibody and of first isolation of JEV from mosquitoes varied from year to year: the 2-ME-sensitive HI antibody might appear first; JEV might be isolated from mosquitoes first; or the two events might occur on about the same date. In some years, the HI assays indicated a two-stepped development of infections in pigs, as in the Miyagi Prefecture in 1964 (above) (Hayashi *et al.*, 1970, 1976; Fukumi *et al.*, 1975). The data suggested that the number of human infections was determined by the population

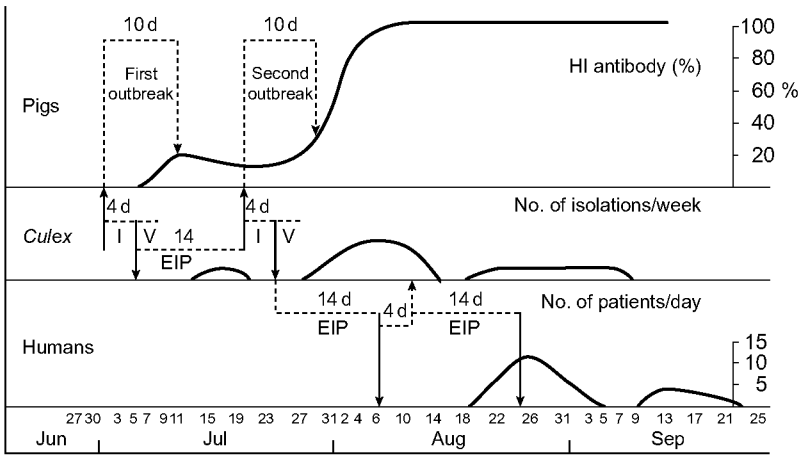


Figure 45.23 Temporal relationships between Japanese encephalitis virus infections in pigs and humans, and isolations of JEV from *Culex tritaeniorhynchus*, in the Miyagi Prefecture of Honshu, Japan during 1964. (After Konno *et al.*, 1966.)
I, 4-day period between inoculation of pigs and start of viraemia; V, 2–4-day length of viraemia; EIP, 14-day duration of extrinsic incubation period in mosquitoes. The time from inoculation of pigs to first measurable haemagglutination inhibition antibody in pig serum was 10 days.

density of *Cx. tritaeniorhynchus* during the epizootic in pigs. Below a threshold vector density, the number of human cases was extremely small; above the threshold, a linear relationship obtained between number of cases and vector density. The immune status of the human population would also influence the number of new cases in any year (Wada *et al.*, 1975).

On **Amami Island** (as noted earlier), during warm winters JEV may survive through continuation of the pig–mosquito cycle, but after cooler winters it must reappear from another source. The period of JEV dispersion varied from year to year, but there appeared to be some temporal association between appearance of the virus in its mosquito and vertebrate hosts. Thus, in 1973, when JEV was isolated from overwintering *Cx. tritaeniorhynchus* during February, and could be isolated from mosquitoes in most months between February and November, pigs were infected in each month from March to December. In contrast, in 1974, when JEV was isolated from *Cx. tritaeniorhynchus* only between mid-July and late October, pigs were infected between mid-July and December (Hayashi *et al.*, 1975).

On **Okinawa**, the population density of *Cx. tritaeniorhynchus* was low and fluctuated substantially, supposedly because the rice fields were small and scattered. Piggeries also were small and scattered, and at all times there were some susceptible pigs (Iha, 1971). In each of the years 1966 to 1976, during a period of 2 or 3 months in the summer, 2-ME-sensitive HI antibody was detected in 20–50% of slaughtered pigs. In 1966, 2-ME-sensitive antibody was recovered from some pigs in every month, but that was exceptional. The first occurrences of JEV in *Cx. tritaeniorhynchus* and in pig sera slightly preceded the appearances of 2-ME-sensitive antibody. Human infections with JEV tended to follow those in pigs (Ura, 1976b).

(d) Disappearance phase

On the **Kantō Plain**, the number of new infections declined in late September, and the virus disappeared until the following summer. This could be partly explained by changes to the amplifying hosts, because by mid-September pigs that had not been slaughtered were immune. At Sagiyama heronry, it was impossible to test ardeids adequately after the first week in September because screening was limited to nestlings and juveniles, so the date when infection of ardeids ceased could not be determined. It was believed that the bird populations were never exhausted of susceptibles, so the cessation of virus dispersion was ascribed to the disappearance of active vectors (Buescher *et al.*, 1959b).

In the **Nagasaki** area, isolation of JEV from *Cx. tritaeniorhynchus* ended during the first days of September at the latest, and females entered diapause apparently uninfected (Hayashi *et al.*, 1966, 1976). On **Okinawa**, during the period 1969–1975, JEV disappeared from the *Cx. tritaeniorhynchus* population during September or October (Ura, 1976a). On **Amani Island**, the date when virus dispersion ended varied. In some winters JEV appeared to persist at a low level in the mosquito–pig cycle (Wada *et al.*, 1973, 1976).

(e) Transport by migrating birds

In early spring in the northern hemisphere, birds of a number of species fly northwards from their subtropical or tropical overwintering sites to their breeding sites in the temperate region. The possibility that some might arrive in Japan while viraemic with JEV has often been postulated as a means of annual reintroduction of the virus. Findings from studies of ardeid birds on the Kantō Plain proved informative.

During the winter months, no ardeids were present at Sagiyama, and only a few hundred little egrets remained at Shinhama. Migration to both sites occurred annually in March and April. Often the birds would appear suddenly, and immigration would be completed within a period of 1 to 2 weeks. Breeding by ardeids began in April and ended in September, but the breeding periods of the species differed. In the years of observation, great egrets and cattle egrets bred from May to early July, and no nestlings were present during late July

and August, when mosquitoes were infected with JEV. Indeed, by mid-July, many cattle egrets had left Sagiyama. In contrast, the breeding periods of the black-crowned night herons, and of intermediate and little egrets, lasted until mid-August, and their nestlings appeared concurrently with the infected mosquitoes (Scherer *et al.*, 1959b).

In 1953 and 1957, mosquitoes were examined for JEV in April, May and early June, and none was found infected. If JEV had been introduced into the Kantō Plain by migrating birds in April, *Cx. tritaeniorhynchus* would not have been available in large numbers until June for transmission of the virus to other ardeids (Buescher *et al.*, 1959a). At Shinhama in 1956, JEV was first isolated from *Cx. tritaeniorhynchus* on 20 July, well before the first isolation from wild ardeids on 8 August (Buescher *et al.*, 1959a,b). Migration from the Kantō Plain begins in early September, when avian infection is still demonstrable in the colonies. It was thought possible that some birds might travel southwards for hundreds of miles before viraemia ceased (Scherer *et al.*, 1959b). Such is the case with white storks (Ciconiidae: *Ciconia ciconia*) infected with West Nile virus (Section 45.7.8.d).

The absence of JEV from ardeids before mid-July, shown by sensitive antibody tests of nestlings at Shinhama and Sagiyama, was inconsistent with the supposed introduction of virus by birds migrating to Japan from South-east Asia 3 months earlier. The facts indicate that ardeid birds do not bring JEV to the heronries each year, but rather that the virus appears first in mosquitoes, which in turn infect the ardeids. If non-ardeid migrant birds were shown to carry JEV, the problem would merit a full reinvestigation.

(f) Overwintering in farmed pigs

An exceptional suggestion concerning the overwintering of JEV came from studies in Hokkaido^{-} Island, the most northerly of the main Japanese islands. In 1984 and 1985, outbreaks of abortion and stillbirth were recorded at seven pig farms in the south of Hokkaido^{-}. From the size of fetuses aborted in June or July 1985, it was estimated that one had been dead *in utero* since the middle of April, and that another had died in late May, before outbreaks of JE in pigs in Honshu Island to the south. Serological tests conducted in 1984–1986 showed anti-JEV HI antibodies to be present in pigs in 11 of 14 districts in the non-mountainous parts of Hokkaido^{-}. Prevalence was highest in two districts in the north-east of the island and two in the south. During 1984–1986 detailed serological surveys were undertaken at farms, one identified as farm A, in a small area near Sapporo city in the south of Hokkaido^{-}. In 1984, positive sera were obtained from four of 11 farms, namely farm A and three farms close to it. In 1985, positive sera were collected from six of 15 pig farms, namely farm A and three nearby farms and, some distance away, from two farms situated close to each other. In 1986, positive sera were obtained from two of 13 farms, namely farm A and one closely adjacent to it.

The overwintering of JEV in Hokkaidō, at the northern limit of its range, is enigmatic. The cases of abortion or stillbirth occurring early in the year, and the seropositivities found in endemic foci, could not be explained by movements of pigs into those areas. *Culex tritaeniorhynchus* was much too scarce to serve as a vector, and no herons or egrets were seen. Takashima *et al.* (1988) concluded that in Hokkaidō JEV overwintered locally in endemic foci, infecting farmed pigs and an unspecified mosquito host, possibly *Cx. pipiens pallens*, which survived the winter in the pig pens. The authors' suggestion that further south in Japan JEV might persist through the winter in endemic foci was inconsistent with the much later annual appearance there of JE in farmed pigs.

(g) Overwintering in reptile hosts

There is no evidence that JEV might overwinter in snakes. No JEV was isolated from sera from 305 wild-caught snakes. Anti-JEV antibody was found in sera from six snakes of three species (n = 207), but the infections were thought not to be epidemiologically significant (Hayashi *et al.*, 1976). Of four species of snake kept at 26°C and inoculated with up to 10^7 (?mouse) LD_{50} JEV, only *Rhabdophis tigrinus* showed an apparent viraemia. No evidence

of replication was observed, and the circulating virus, which decreased rapidly in titre from the first to third day, was thought to be surviving inoculum (Oya *et al.*, 1983).

After inoculation of five species of lizard with up to $10^{4.7}$ LD_{50} JEV, viraemias were observed in four species (*Takydromus tachydromoides*, *Eumeces latiscutatus*, *Eumeces barbouri* and *Eumeces marginatus oshimensis*) for up to 5 days post-inoculation. The minimum infective dose was *c.* 10^3 LD_{50}, comparable to the amount injected in a single mosquito bite. In *E. latiscutatus*, virus disappeared rapidly from the blood after inoculation but reappeared later, at 26°C peaking at 6 days, and at 21°C peaking at 14 days post-inoculation. Ingestion of only a single infected mosquito by individual *E. latisculatus* was sufficient for transmission of JEV (Oya *et al.*, 1983).

To find whether lizards might serve as overwintering hosts of JEV, experiments were carried out with *T. tachydromoides* and *E. latiscutatus*, using *Cx. pipiens* and *Cx. quinquefasciatus*, which fed readily on lizards, as vectors. Transmission of JEV from infective mosquitoes to uninfected lizards by bite, and from infected lizards to a healthy mouse by vector mosquitoes was demonstrated. JEV replicated in lizards kept at 26°C, disappeared from them several days after transfer to 10°C, and could be isolated again from lizards returned to 26°C.

To simulate natural hibernation, lizards were kept at temperatures that were (or simulated) the natural daily maximum and minimum outdoor temperatures throughout the winter. Lizards inoculated with JEV on 14 October 1968 were observed to enter into hibernation on 19 October and to emerge from hibernation on 10 April 1969. Viraemias were detected during a few days in late April 1969. To find whether wild-caught reptiles might be infected with JEV, blood samples from seven species of snake and three species of lizard were examined for viraemia and submitted to HI assay. None of the many individuals tested was viraemic, but positive results to the HI assay were obtained in 14.3% of *E. latiscutatus* samples (n = 100) and 4.0% of *T. tachydromoides* samples (n = 73) (Doi *et al.*, 1983). It appears that JEV might overwinter in certain lizards, but that, even so, any contribution to its perpetuation might be slight.

45.4.8 Effects of intervention in Japan

(a) Modes of intervention

During the later decades of the 20th century, three different approaches were used to protect the Japanese population and certain domesticated animals from Japanese encephalitis: (i) reduction of vector populations; (ii) protection of farmed pigs, the amplification host; and (iii)–(iv) immunization of horses and humans, both dead-end hosts.

(i) The widespread treatment of irrigated rice fields with organophosphate larvicides appeared to reduce the larval and pupal densities of *Cx. tritaeniorhynchus*, but decreases in vector density also occurred in localities where larviciding was not practised. Certain herbicides that had been used on rice, e.g. nitrofen, showed modest larvicidal activity and part of the reduction of *Cx. tritaeniorhynchus* density in rice fields was ascribed to the larvicidal actions of those herbicides or to their undefined effects on the biotic community (Maeda *et al.*, 1981).
(ii) The risk to farmed pigs of infection with JEV was reduced by modifications of animal husbandry: the birth of new susceptibles was restricted to certain periods of the year; pig pens were moved to isolated locations, far from rice fields; and the distances between human habitations and sites of the pig–*Culex* transmission cycle were increased. Immunization with attenuated live vaccine blocked infection in pigs bitten by infective mosquitoes, but two difficulties limited the use of immunization: the high turnover rate necessitated immunization of large numbers of newborn pigs each year; and the presence of maternal antibodies restricted the period when the vaccine could be used (Igarashi, 2002).
(iii) An inactivated JE vaccine for horses was introduced in 1948 (Konishi *et al.*, 2004).
(iv) A vaccine for the protection of human populations against JE was first introduced in 1954, since when three types have been used: (a) inactivated vaccines derived from mouse brain; (b)

inactivated vaccines cultivated on primary hamster kidney cells; and (c) live attenuated vaccine cultivated on primary hamster kidney cells (Yun and Lee, 2006; Wilder-Smith and Halstead, 2010).

(b) Effects of intervention on the prevalence of JE and the enzootic transmission cycle

The effectiveness of interventions in JEV transmission in Japan was shown by the reductions in prevalence of JE in two dead-end hosts. *Horses.*The number of cases of equine JE fell during the 1950s and early 1960s, and between 1968 and 2003 no equine case was reported (Konishi *et al.*, 2004). *Humans.* Following the initiation in 1967 of the nationwide distribution of a high-purity inactivated JE vaccine, the annual number of human JE cases declined – from >1000 before 1967 to <10 after 1992 (Table 45.18) (Igarashi, 2002). Away from the non-endemic northern areas, annual infection rates in humans ranged from 3–17% before 1960 to 5–10% in the early 1980s and mid-1990s, to 0.2–3.4% between 2001 and 2004 (Kurane, 2002).

Pigs. Changes in the husbandry and immunization of farmed pigs were thought to have diminished the amplification of JEV and so its transmission to humans. Chemical treatment of rice fields was thought to have reduced the population densities of *Cx. tritaeniorhynchus*, and so to have reduced the transmission of JEV from pigs to humans. In Kyoto City, during the period 1965–1973, a statistically significant correlation was obtained between the estimated size of the adult *Cx. tritaeniorhynchus* population, as measured by light trapping, and the number of JE cases (Table 45.19) (Maeda *et al.*, 1978). Whether these correlations, or claims of cause and effect, were epidemiologically important is uncertain. Correlations between aspects of the biology of a host or vector and an infectious agent that are statistically significant are not necessarily epidemiologically significant.

It was of interest to know the extent to which human populations protected against JE by vaccination were affected by the continuing enzootic transmission cycle of JEV. Human serum samples were screened for antibodies to the JEV non-structural protein NS1; such antibodies are induced by infection with JEV but not by inoculation with the inactivated vaccines, which contain only structural proteins. The screening revealed that, during years when the prevalence of JE was low, relatively high proportions of the population were seropositive for JEV-specific antibodies (Konishi and Suzuki, 2002).

Similar findings were obtained with equines. Serological data for the year 2000 are representative

Table 45.18 The incidence of Japanese encephalitis in Japan during the five decades between 1950 and 1999 as: (i) the number of clinical cases of JE in humans; and (ii) the number of deaths from the disease. (From the data of Igarashi, 2002.)

Decade	*No. of cases p.a. (range)*	*No. of deaths p.a. (range)*
1950–1959	1729–5196	720–2430
1960–1969	230–2638	228–1500
1970–1979	4–145	9–167
1980–1989	21–44	5–20
1990–1999	2–55	0–10

The anomaly in the data for 1970–1979 of the number of deaths p.a. exceeding the number of cases is as reported without comment by Igarashi (2002).

Table 45.19 The number of clinical cases of Japanese encephalitis and the total number of *Culex tritaeniorhynchus* captured by light traps in each year from 1965 to 1973 in Kyoto City. (From Maeda *et al.*, 1978.)

Year	*No. of patients*	*Culex tritaeniorhynchus*	
		Total catch	*MPI* *
1965	44	9,438	117.2
1966	96	8,665	215.9
1967	41	11,570	100.0
1968	10	2,566	25.6
1969	16	2,583	40.4
1970	0	544	9.0
1971	0	338	3.3
1972	0	182	1.5
1973	0	175	2.7
Coefficient of correlation with number of patients		0.808	0.997

*, Mean per cent index. Calculated to adjust for the relative abundance of mosquitoes at different collecting stations relative to the year 1967.

of those obtained over a longer time period. In Hokkaidō, the mean seropositivity rate for NS1 was 25% (n = 36); at four locations in central Japan it was 16–67% (n = 138); and in Saga, southern Japan, it was 50% (n = 38). Comparison of antibody titres in 2- and 3-year-old horses suggested that they had been boosted by exposure to JEV over two epizootic seasons (Konishi *et al.*, 2004).

These high seropositivity rates show that in Japan the human and equine populations continue to be attacked by infective mosquitoes, and that immunization prevents viral infections developing in dead-end hosts bitten by infective mosquitoes. JEV continues to be maintained in its natural reservoir hosts throughout Japan.

(c) Later developments

Reports of post-vaccination medical problems in humans raised opposition to vaccination, and in 2005 the Japanese government withdrew its strong recommendation for JE vaccination. In 2007. only about 10% of children aged 3–4 years were inoculated. However, the number of reported JE patients remained low in situations where a relatively large number of unvaccinated children had been exposed to natural JE infection. With no supporting evidence, it was surmised that the form of JEV circulating in Japan had become less virulent.

Production of the inactivated mouse-brain-derived JE vaccine remains discontinued in Japan. However, a new inactivated Vero cell-culture-based JE vaccine, IC51, has been licensed in Europe and the USA, and other countries are in the process of licensing the vaccine.

In some Asian countries, non-vaccine control of human JE is virtually impossible because it requires a multifactorial approach, including sentinel animal surveillance, changes to agricultural practices and vaccination of pigs (Konishi *et al.*, 2010; Wilder-Smith and Halstead, 2010).

45.4.9 Transmission cycles and seasonality in Sarawak

Sarawak (now a state of Malaysia), which occupies most of the north-west of the island of Borneo, provides an example of Japanese encephalitis in the humid tropics. Initial short-term investigations in different regions of the country, carried out between 1961 and 1966, had shown *Culex tritaeniorhynchus* to be present in substantial numbers everywhere. *Culex gelidus* was primarily associated with pigs, and to a lesser extent with poultry and cattle (Macdonald *et al.*, 1965, 1967). In a search for arboviruses, 166,000 mosquitoes were collected over an extensive area where little primary rain-forest remained as a result of the traditional practice of shifting cultivation. Analysis of pools of mosquitoes revealed a number of different arboviruses, notably in three *Culex* species – *Cx. tritaeniorhynchus, Cx. gelidus* and *Cx. pseudovishnui*. JEV was recorded at minimum infection rates of 0.25/1000 in *Cx. tritaeniorhynchus* and of 0.16/1000 in *Cx. gelidus*; it was not found in *Cx. pseudovishui* (Simpson *et al.*, 1970b). Analyses of human sera showed anti-JEV antibodies in all areas, with a mean prevalence rate of 6% (Smith *et al.*, 1974).

(a) The location

More intensive investigations were undertaken between October 1968 and February 1970 in a single village and rice-field ecosystem. Kampong Tijirak (1° 20' N, 110° 24' E), on the wide coastal plain, was a Land Dyak (Bidayuh) village of 80 dwellings of wood and palm-thatch construction, and some 500 inhabitants (Figure 45.24). To the south of the village was a long-established rice field of approximately 16 ha, subdivided into some 400 plots, from which a single rice crop was produced each year. The plots were flooded from September to the end of January, and then dried off before the crop flowered. Harvesting took place during March and April, after which the plots were left fallow until planting in September. Water retained in the plots could be fresh or stagnant; when stagnant it often had a rust-coloured precipitate of ferric oxide and a brown surface scum (Heathcote, 1970). In this region, the annual rainfall is often 300–400 cm per annum. From October until January or February, the north-west monsoon brings much rain; the south-east monsoon, from April until July

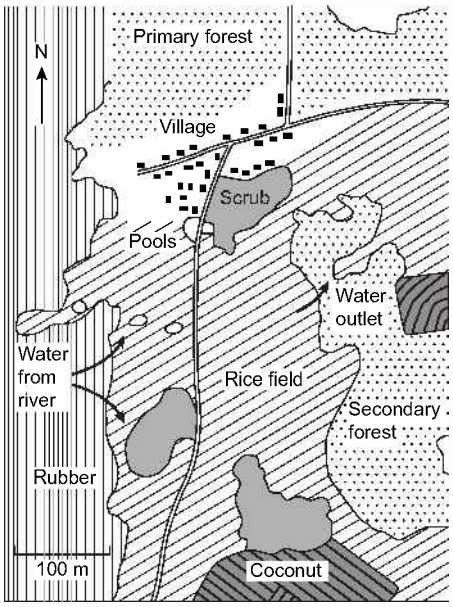


Figure 45.24 Map of Tijirak Village, Sarawak, and the surrounding area (c. 1970). To the south of the village was an extensive wet rice field of an estimated 16 ha, part of which is shown. To the west was a rubber plantation, but otherwise the village and the rice field were surrounded by primary and secondary forest and scrub. The rice field was divided into many plots, with an average size of about 320 m^2. Water from a small river on the west side entered from two outlets and fed all of the rice field, but because the land sloped gently west to east, only about 100 plots retained water long enough to provide larval habitats. The plots were flooded from September to the end of January and then dried. The secondary forest scrub situated between the village and rice field contained pools at certain times of year. A track ran through the village and rice field. (Redrawn from Heathcote, 1970; and Hill, 1970.)

or August, is less productive (Macdonald *et al.*, 1965).

A number of small piggeries were present in the area, but within Tijirak no more than nine pigs were kept at any one time. Limited studies were undertaken in nearby villages and in two large piggeries, which were several kilometres distant and situated away from rice fields. Sporadic collections were made at the so-called '6½-mile piggery', a large piggery some 12–13 km from Kampong Tijirak. In populated parts of Sarawak, wildlife had been hunted almost to extinction (Heathcote, 1970; Simpson *et al.*, 1970a,b, 1974).

(b) The mosquitoes

Monthly sampling of mosquito larvae along a transect that ran through the rice field, the village and into the primary forest captured ten species of *Culex* (including *Cx. tritaeniorhynchus*, *Cx. gelidus* and *Cx. pseudovishnui*) and species of ten other genera. The larval habitat most favoured by *Cx. tritaeniorhynchus* was water to which fresh organic matter was constantly being added, that was becoming stagnant and that had a surface scum. Such habitats were produced in ditches by pollution from pig faeces or outflow from houses, and in irrigated rice fields by infusions of cut vegetation. From May to September, when the paddy area was unsuitable, *Cx. tritaeniorhynchus* larvae were found in rusty coloured pools in the scrub forest. *Culex pseudovishnui* larvae were found in contaminated pools and in pools that were about to dry up. *Culex gelidus* larvae were found in rice plots, especially before planting. Fish ponds contained almost no mosquito larvae (Heathcote, 1970).

Sufficient natural larval habitats were available to enable these mosquitoes to breed throughout the year, but population densities were affected by agricultural practices. In January, the plots were flooded, but few larvae could be found in these sheets of fresh water. In February and March, after the plots had been dried out, there were a mass of very small temporary pools with populations of *Cx. pseudovishnui* and *Cx. tritaeniorhynchus* larvae (10:1). In March and April, after harvesting, most plots dried out completely, but about twelve remained flooded and produced *Cx. tritaeniorhynchus* and *Cx. pseudovishnui* for a further 2 months. Population growth was greatest before rice planting. During September and October, the vegetation on the rice plots was scythed and left lying, producing pools polluted with an infusion of the vegetation. During the following 2 days there was very heavy oviposition by *Cx. tritaeniorhynchus* and, in places, by *Cx. gelidus* and *Cx. nigropunctatus*, and it was calculated that an average-sized plot produced up to

30,000 adults daily for 3–5 days. Planting was spread over about 6 weeks, during which there were always plots to act as population amplifiers. The heavy rains of the NE monsoon that fell in the latter part of December had no substantial effect on the *Cx. tritaeniorhynchus* population size (Heathcote, 1970).

A total of 280,000 adult mosquitoes was collected in and around Kampong Tijirak, from human bait catches at six sites, and from light traps in three piggeries. The species composition was: *Cx. tritaeniorhynchus*, 62.5%; *Cx. gelidus*, 6.6%; *Anopheles* spp., 12.5%; *Mansonia* spp., 10.2%; other species, 8.2%. *Culex tritaeniorhynchus* was the principal species in the rice-field habitat. In a small-scale analysis of 213 blood meals from *Cx. tritaeniorhynchus*, 90.1% were from pigs, 5.2% from dogs, 3.7% from humans, 0.5% from unidentified mammal and 0.5% from birds (Hill, 1970). Possibly the feeding rate recorded from birds was unrealistically low and unrepresentative of wild birds. When geese were presented as bait, *Cx. tritaeniorhynchus* formed 49% of the catch (n = 309) (Bendell, 1970). *Culex tritaeniorhynchus* fed at night. Feeding on human bait and at piggeries began between 18.00 h and 19.00 h, and a fairly constant level of biting activity was maintained until dawn. The human population received some protection by being indoors at night and through use of bed nets. *Culex gelidus* occurred in large numbers in association with pigs, especially where the piggeries were away from rice fields; it fed at night and mainly on pigs. *Culex pseudovishnui* was not captured in human-baited traps or in light traps in piggeries (Bendell, 1970; Hill, 1970; Simpson *et al.*, 1974).

(c) *Virology and serology*

Between November 1968 and December 1969, approximately 158,500 mosquitoes collected at Kampong Tijirak, and 15,800 collected at the '6½-mile piggery', were screened for ten arboviruses. Of 985 pools of *Cx. tritaeniorhynchus* from Kampong Tijirak, arbovirus was present in 35; and, of 23 of those pools that were tested further, JEV was present in four. Based on a pool size reported as 'approximately 100', the MIR would have been of the order of 1.7/1000. None of 19 pools of *Cx. gelidus* from Kampong Tijirak was positive for arbovirus. JEV was not isolated from *Cx. pseudovishnui*.

Of 268 human serum samples from all age groups, collected at Kampong Tijirak during October and November 1969, 63% had anti-JEV antibody. From this figure, an annual infection rate with JEV of approximately 6% was estimated. The percentage of humans having NT and HI antibodies increased with age, reaching 80% in the 15–20-year age class. The estimated infection rate of 6% p.a. was much lower than that in pigs (see below), supposedly owing to the lower attack rate of *Cx. tritaeniorhynchus* on humans.

At the '6½-mile piggery', one of 128 pools of *Cx. gelidus* contained JEV, but no JEV was isolated from 26 pools of *Cx. tritaeniorhynchus*. Blood samples taken from pigs throughout 1969 showed that the proportion with anti-JEV neutralizing antibody varied with age. At 1 month old, 17% were seropositive, but this proportion declined to 0.6% at 3 months old, presumably owing to loss of maternal antibody. The proportion with antibody increased with age thereafter, at an estimated rate of 17% per month. The 50% antibody-conversion age was found to be 7½ months (± 2 months s.d.). Ninety per cent of pigs would have acquired antibody as a result of natural infection by the age of 12 months. In fact, most pigs were slaughtered by the age of 6 months, and there was a continuous production of fresh, susceptible pigs. The prevalence of antibodies in pigs increased considerably during the last quarter of the year, coincident with a seasonal peak in size of the *Cx. tritaeniorhynchus* population (Simpson *et al.*, 1970a).

Blood samples showed 18% of wild birds to have neutralizing antibody to JEV. Larger paddy-field species identified as watercock and bittern accounted for almost one-half of those with antibody. Anti-JEV neutralizing antibodies were present in six of 31 ducks and 16 of 19 dogs; none were found in geese, fowl or rodents. Because antibodies in birds were considered to be labile, Simpson *et al.* (1970a) suggested that a larger

proportion of wild birds may have been infected with JEV than their results indicated.

45.4.10 Summary

(1) As with the other members of the Japanese encephalitis virus group (where known), the amplifying hosts of JEV are wild birds and the vectors are species of *Culex* (*Culex*). Birds of the family Ardeidae are the most important known amplifying hosts of JEV. The other amplifying hosts are feral and farmed pigs.

(2) The wide and increasing distribution of JEV in Asia can be ascribed to characteristics of its vectors and of its amplifying host. A number of species of *Culex* (*Culex*) use rice fields as larval habitats, where huge populations are produced. The adult females bite both birds and mammals, and are readily infected by JEV. In large parts of the geographical range of JEV, *Cx. tritaeniorhynchus* is the main enzootic vector and the main bridge vector. *Culex vishnui* is a putative main vector in South India. *Culex gelidus* is important in maintaining the mosquito–pig transmission cycle in Sarawak, and it is a local vector on islands in the Torres Strait. *Culex annulirostris* is a putative local vector in Papua New Guinea, on islands in the Torres Strait and in Cape York (Queensland).

(3) Farmed pigs are an important source of blood for the vector species, enabling the build-up of their populations, and they are important amplifying hosts of JEV throughout most of its geographical range. Farmed pigs occur abundantly in many countries, where rearing and slaughter practices ensure a steady supply of susceptible young. Without the involvement of pigs in amplification cycles, transmission to humans would rarely occur. Humans and horses are dead-end hosts. Humans affect the ecology and epidemiology of JEV through the irrigation of rice fields and pig farming; possibly, before these relatively recent developments, JEV cycled only between birds and mosquitoes.

(4) At all latitudes JEV transmission is seasonal. At higher latitudes, i.e. in cool-temperate regions, the way in which JEV survives through hard winters and becomes available to start summer amplification cycles is uncertain. There is no direct evidence of its overwintering in vertically infected, hibernating mosquitoes (Section 44.6.2.b), or that it is carried by infected wind-borne mosquitoes from southern latitudes. Carriage in returning migratory birds appears improbable; where examined, the adult birds were immune or non-infected. *Culex tritaeniorhynchus* hibernates during the winter as adult females, but it is uncertain whether the large numbers of females that appear in early spring, and that will be the parents of the early summer generation, have survived hibernation or have been carried in by winds from the south.

(5) As latitude decreases, JEV infections appear progressively earlier in the year and the transmission season is longer. In subtropical locations and during warmer winters, there is evidence of mosquito–pig transmission continuing into the winter. On the subtropical Ryuku archipelago, female *Cx. tritaeniorhynchus* remain in diapause only briefly, and the females may blood feed on warmer winter days. On some of these islands, JEV may persist through milder winters in the mosquito–pig cycle. It has not been established how JEV reappears after cooler winters.

(6) In tropical regions, transmission depends on the availability of aquatic larval habitats, which is governed by seasonal patterns of rainfall or irrigation. Findings from Sarawak showed that JEV was maintained throughout the year in a transmission cycle involving farmed pigs as amplifying hosts and *Cx. tritaeniorhynchus* and *Cx. gelidus* as vectors. A variety of larval habitats enabled the two vector species to breed throughout the year, while agricultural practices that produced additional larval habitats permitted seasonal increases in vector populations.

(7) In certain countries, including Japan, intervention measures such as changes of agricultural practice, the mass vaccination of children and vector control programmes have reduced human cases of JE to very small numbers. Even so, in Japan, JEV still appears in its natural amplifying hosts and in *Cx. tritaeniorhynchus* as vector. In large areas of South and South-east Asia, Japanese encephalitis remains a serious problem of human health.

45.5 TRANSMISSION OF LA CROSSE VIRUS

45.5.1 La Crosse virus

La Crosse virus (LACV) is a strain of *California encephalitis virus*, a species of the genus *Orthobunyavirus*, family *Bunyaviridae*. *California encephalitis virus* (CEV) was first isolated in 1943 and 1944 from pools of *Ochlerotatus melanimon* (cited as *Ae. dorsalis*) collected in Kern County, California and in 1944 from a pool of *Culex tarsalis* also from Kern County (Hammon and Reeves, 1945; Hammon *et al.*, 1952). Later, it was established that CEV comprises 13 antigenically related and named viruses, for many years described as serotypes but now classed in the ICTV database as strains (Fauquet *et al.*, 2005). As a group they are widely distributed, but individual strains are endemic within definable geographical areas. Ten strains occur in North America, including San Angelo virus which is known from Texas, and snowshoe hare virus which occurs in arctic and subarctic North America. Four strains occur in other regions of the world: Melao virus in the Caribbean and South America; Serra do Navio virus in South America; Tahyna virus in Africa, Asia and Europe; and Inkoo virus widely in Europe (LeDuc, 1979). HI antibodies to California antigenic group viruses were found in five residents (n = 126) of a suburb of Shanghai, where the highest neutralization titres were to snowshoe hare virus (Gu *et al.*, 1984). Species of *Ochlerotatus*, *Culex*, *Culiseta* and *Psorophora* are the putative vectors, and small mammals provide the vertebrate hosts of most CEV strains. However, deer appear to be the amplifying host of Jamestown Canyon virus (LeDuc, 1979; Boromisa and Grimstad, 1987).

La Crosse virus is endemic in small areas of hardwood forest in most states that are east of or that adjoin the Mississippi River. The principal amplifying hosts are sciurid rodents, and the main vector is the tree-hole mosquito *Ochlerotatus triseriatus*. Humans are dead-end hosts. The first isolation of LACV was from the brain tissue of a 4-year-old girl who lived on the west bank of the Mississippi River, 8 miles north of La Crosse, Wisconsin, and who died with meningoencephalitis (Thompson *et al.*, 1965). LACV is the most severely pathological strain of CEV, and it continues to be a major cause of encephalitis and aseptic meningitis in children in most states east of or contiguous with the Mississippi River. Like that of the virus, the distribution of cases corresponds to regions in which hardwood deciduous forests predominate. Infections occur principally in children, and particularly in boys (male to female ratio 1.8:1), which possibly reflects their greater exposure to infected mosquitoes (McJunkin *et al.*, 1998). LACV has also been isolated from populations of *Oc. triseriatus* in New York State and Connecticut (Armstrong and Andreadis, 2006). The disease is sometimes, but only rarely, named 'La Crosse encephalitis' in medical journals (McJunkin *et al.*, 1998, 2001).

Analysis of the relatedness of 23 strains of LACV by oligonucleotide finger printing of the RNA genomes showed them to fall into three classes – Types A, B and C. Type A strains had RNA sequences closely related to those of the prototype LACV, and were recovered from Wisconsin, Minnesota, Indiana and Ohio. Type B strains had RNA sequences which, although related to those of Type A, were distinct from them; they were recovered from Minnesota, Wisconsin and Illinois. Type C strains had RNA sequences that resembled neither the A nor the B type sequences closely; they were recovered from eastern Ohio, New York State, Texas, Georgia and North Carolina (Klimas *et al.*, 1981).

Additional information on La Crosse virus can be found in Chapter 44: RNA segment reassortment (Section 44.1.1.c); venereal transmission (Section 44.5); laboratory studies of vertical transmission (Section 44.6.1.a); inheritance of factors controlling vertical transmission (Section 44.6.3); infection, multiplication and dissemination in mosquito hosts (Section 44.8); pathology of infection in vertebrates (Section 44.10.2.b).

45.5.2 Vertebrate hosts

(a) Classification

Rodentia, Sciuridae
- *Sciurus*: *S. carolinensis* (Eastern grey squirrel), *S. niger* (Eastern fox squirrel)
- *Tamias*: *T. striatus* (Eastern chipmunk)

Artiodactyla, Cervidae
- *Odocoileus*: *O. virginianus* (white-tailed deer)

Carnivora, Canidae
- *Urocyon*: *U. cinereoargenteus* (grey fox)
- *Vulpes*: *V. vulpes* (syn. *V. fulvus*, red fox).

In addition to the species listed above, the Northern raccoon (*Procyon lotor*, Procyontidae) is an occasional host of LACV, neutralizing antibodies being found in a low percentage of wild raccoons, but it is thought not to be a significant host (Amundson and Yuill, 1981).

(b) Virology and serology of LACV in wild mammals

In areas where LACV is endemic, the virus has been isolated from Eastern chipmunks (*Tamias striatus*), two tree squirrels (*Sciurus carolinensis* and *S. niger*), and the red fox (*Vulpes vulpes*). LACV-specific neutralizing antibodies have been found in blood samples from chipmunks, Eastern grey squirrels, Eastern fox squirrels, white-tailed deer, red and grey foxes, and woodchucks (also known as the groundhog, *Marmota monax*). Among chipmunks born in spring or summer, most infections occurred during the following late summer and autumn, ceasing after the arrival of freezing temperatures (Moulton and Thompson, 1971; Issel *et al.*, 1972b; Gauld *et al.*, 1975; Amundson and Yuill, 1981; Amundson *et al.*, 1985).

Rodents. In the laboratory, when chipmunks were infected by the bite of infective *Oc. triseriatus*, viraemias were detected on days 1–7 post-exposure, with the mean titre peaking at $10^{6.8}$ SMIC (suckling mouse intracerebral) LD_{50} on day 2. Intramuscular inoculation of eight chipmunks with $10^{4.3}$ SMIC LD_{50} LACV resulted in viraemias that were of significantly lower titre and shorter duration, and the viraemias were measurable only on days 1 and 2 post-infection, with a maximum mean titre on day 2 of $10^{2.8}$ SMIC LD_{50} (Osorio *et al.*, 1996). Wild-caught Eastern chipmunks were classed as juvenile, subadult or adult, and exposed to *Oc. triseriatus* vertically infected with LACV. Blood samples taken daily for 5 days post-infection showed infection in 16/16 juveniles, 13/17 subadults and 21/29 adults. For juvenile, subadult and adult chipmunks, the mean viraemias were $10^{4.6}$, $10^{4.5}$ and $10^{4.8}$ SMIC LD_{50} ml^{-1}, respectively, and the average durations of viraemia were 2.4, 2.3 and 2.4 days, respectively (Figure 45.25). Any differences between age classes for titre and duration were not significant (Patrican *et al.*, 1985a).

When female *Oc. triseriatus* fed on viraemic chipmunks, the percentage of mosquitoes that became infected was proportional to the titre of the viraemia, rising to 100% infected as the viraemia increased from $10^{3.8}$ to $10^{5.2}$ SMIC LD_{50} ml^{-1} (Table 45.20). Regression analysis indicated a 50% infectious dose (ID_{50}) for the mosquitoes to be $10^{4.87}$, with 95% confidence limits of $10^{4.73}$ and $10^{4.92}$. After an extrinsic incubation period of 14 days the mosquitoes fed on suckling mice, when the likelihood of virus transmission to the mice was again proportional to the LACV titre in the viraemic chipmunks, but with a higher threshold (Patrican *et al.*, 1985b). In other experiments, chipmunks were infected with LACV by the bites of vertically infected *Oc. triseriatus*, when on days 2 and 3 post-infection about half of the viraemias exceeded the minimum titres needed to infect some or most mosquitoes (Figure 45.25) (Patrican *et al.*, 1985a).

Within a broad enzootic area in south-western Wisconsin, the seroprevalence of LACV-specific antibodies was not uniform. Seroprevalence rates

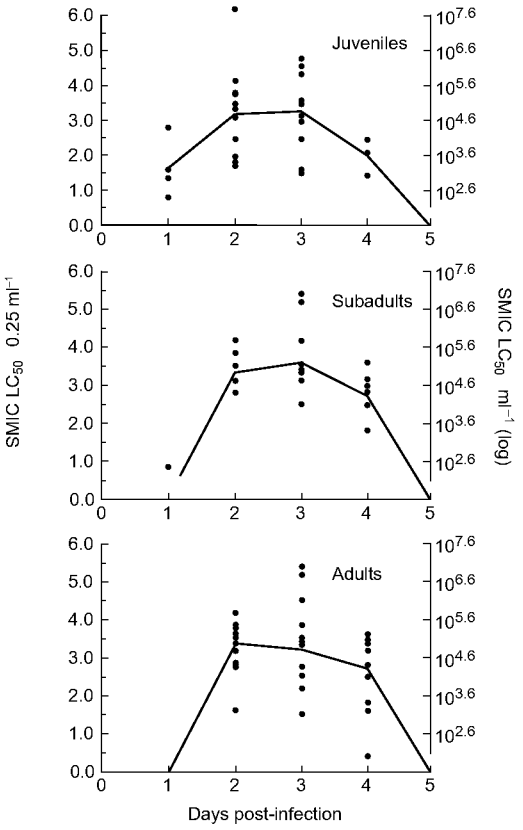


Figure 45.25 Viraemia titres in wild-caught juvenile, subadult and adult chipmunks (*Tamias striatus*) that had been infected with La Crosse virus by the bite of vertically infected *Ochlerotatus triseriatus*. (After Patrican *et al.*, 1985a.) The left-hand y-axis shows the units of measurement used by the investigators to record virus titres ($SMIC_{50}$ (50% suckling mouse intracerebral) 0.25 ml^{-1}); the right-hand y-axis gives those measurements normalized to units ml^{-1}.

were greater in 'high quality' chipmunk habitats than elsewhere. Neutralizing antibodies of maternal origin were found at very low titre in trapped juvenile chipmunks until early July. From that time on, the titre of neutralizing antibodies produced in response to infection rose progressively until mid-September; at one location, seroprevalence rates in spring-born juvenile and adult chipmunks reached 100% by September (Gauld *et al.*, 1974).

Eastern grey and Eastern fox squirrels have not been studied as intensively as chipmunks. At one location in Wisconsin, when seroprevalence rates

Table 45.20 Transmission of La Crosse virus from viraemic chipmunks (*Tamias sciurus*) to females of *Ochlerotatus triseriatus* that fed on them and, after an extrinsic incubation period of 14 days, transmission from those mosquitoes to suckling mice. (From Patrican *et al.*, 1985b.)

Chipmunk viraemia Log_{10} SMIC LD_{50}* ml^{-1}	*Transmission to mosquitoes* (%)	*Transmission to suckling mice* (%)
$10^{6.2}$	100	100
$10^{5.4}$	100	89
$10^{5.2}$	100	83
$10^{5.1}$	85	79
$10^{4.8}$	40	20
$10^{3.8}$	11	0
$10^{2.2}$	0	0

*, Suckling mouse intracerebral LD_{50}

in juvenile, subadult and adult chipmunks were 53% (n = 117), those in both grey squirrels (n = 140) and fox squirrels (n = 36) were 39%. After inoculation with LACV, viraemias were of somewhat higher titre and longer duration in chipmunks than in grey squirrels (Yuill, 1983).

Foxes. A single, naturally infected, sentinel red fox had a viraemia of $10^{3.0}$ SMIC LD_{50} ml^{-1} (Amundson and Yuill, 1981). Each of five red foxes exposed to the bite of a single LACV-infected *Oc. triseriatus* became infected. The viraemias lasted for 4 or 5 days, and the maximum titres ranged from $10^{2.5}$ to $10^{3.7}$ SMIC LD_{50} ml^{-1}. When foxes with maximum viraemia titres of $10^{2.9}$, $10^{3.2}$ and $10^{3.5}$ were exposed to *Oc. triseriatus*, 5%, 10% and 20% of the mosquitoes, respectively, became infected. Those that had fed on the foxes with the two highest viraemias transmitted the virus to chipmunks (Amundson *et al.*, 1985). Three of five foxes that consumed viraemic chipmunks became infected and developed viraemias of similar titre to those in foxes bitten by vectors (Yuill, 1983).

Deer. During the years 1969 to 1971, sera from white-tailed deer killed by hunters in three areas of Wisconsin were screened for specific neutralizing antibodies to two CEV strains – La Crosse virus and Jamestown Canyon virus (JCV). In the south-west area, seropositivities for LACV and JCV in adult white-tailed deer were 17.7% and 23.1%,

respectively. In the west-central and east-central areas, seropositivities for LACV were zero, and for JCV were 76.4% and 90.5%, respectively (Issel *et al.*, 1972b). Some 10 years later (1979–1980), in central Wisconsin, LACV-specific antibodies were found in 7% of adult white-tailed deer (Murphy, 1989).

Viraemia was detected in seven of 11 white-tailed deer inoculated with LACV. The viraemias lasted only 1 day, with peak titres ranging from a trace to $10^{2.6}$ SMIC LD_{50} ml^{-1} (Issel *et al.*, 1972a). When five white-tailed deer were exposed to the bites of LACV-infected *Oc. triseriatus*, two developed viraemias. In one the viraemia lasted 2 days and peaked at $10^{2.3}$ SMIC LD_{50} ml^{-1}, and in the other it lasted 4 days, peaking at $10^{3.0}$ SMIC LD_{50} ml^{-1}. However, *Oc. triseriatus* that fed on the two viraemic animals did not become infected. Also, when the viraemic animals were exposed to 20–25 uninfected *Oc. triseriatus* daily, none of the mosquitoes became infected (Osorio *et al.*, 1996).

Conclusions. Strong evidence that chipmunks and Eastern grey squirrels are amplifying hosts of LACV is provided by the following aspects of their interactions: (i) the close association of chipmunks and Eastern grey squirrels with the main vector of LACV, *Oc. triseriatus*; (ii) the timing of the rodents' infection with LACV; (iii) the viraemias that develop in infected individuals; and (iv) the experimental transmission of LACV to and from those two rodent species. Probably, the red fox can be an amplifying host, although a population density of six foxes per square mile, about one per 45 ha, was considered high (Jackson, 1961), so the red fox can be of only minor importance. White-tailed deer are attacked by *Oc. triseriatus*, and in some locations show significant LACV-specific seropositivity rates, but the absence of infection from females that had fed on viraemic deer suggests that deer are dead-end hosts and a substantial drain on circulating LACV.

45.5.3 Mosquito hosts: classification

Subfamily Culicinae: Tribe Aedini

Ochlerotatus: *Oc.* (*Protomacleaya*) *triseriatus*, *Oc.* (*Ochlerotatus*) *canadensis*

Stegomyia: *St. albopicta*.

The four species of the Triseriatus Group of *Ochlerotatus* (*Protomacleaya*) – *Oc. triseriatus*, *Oc. hendersoni*, *Oc. brelandi* and *Oc. zoosophus*, were reclassified by Reinert *et al.* (2009) as '*Ochlerotatus*' *sensu auctorum*, and are among the many species of *Ochlerotatus* Lynch Arribálzaga that they were unable to assign to a genus.

All four species are tree-hole mosquitoes (Munstermann *et al.*, 1982). Outline maps of the distribution of these species are provided by Darsie and Ward (2005). *Ochlerotatus triseriatus* occurs in the eastern and central-eastern states; *Oc. hendersoni* occurs sympatrically with *Oc. triseriatus* over that region, but its range extends much further to the west. *Ochlerotatus zoosophus* occurs in south-central USA and Mexico, its range partly overlapping those of *Ochlerotatus triseriatus* and *Ochlerotatus hendersoni*. *Ochlerotatus brelandi* occurs in a limited area just outside the ranges of the other three species. Zavortink (1972) reported it from the Chisos Mountains in SW Texas.

Ochlerotatus triseriatus and *Oc. hendersoni* are anatomically indistinguishable from one another, but can be distinguished by PCR amplification of their rDNA and subsequent comparison of their RFLP (restriction fragment length polymorphism) patterns (Reno *et al.*, 2000). *Ochlerotatus hendersoni* is susceptible to infection with LACV, but has a salivary gland barrier and is not considered a vector of that virus (Section 44.8.3.b). Little is known of the other two species.

45.5.4 Biology and vector status of *Ochlerotatus triseriatus*

(a) Distribution and habitat

The tree-hole mosquito *Oc. triseriatus* is broadly distributed throughout central and eastern North America, ranging from Florida and Texas in the south to Maine and Saskatchewan in the north – from 26° N to 50° N (Sims, 1982). Most states with a high incidence of La Crosse encephalitis contain areas of oak, hickory or other climax forest that provide the microhabitats needed for the aquatic stages of this mosquito. Tree holes suitable for

oviposition are especially abundant in oak forests (Woodring *et al.*, 1996). However, in West Virginia, the population densities were higher in 'sugar maple/red maple habitats' than in 'hemlock/mixed hardwood habitats'. In mixed woodland, the number of tree holes may be small. In a survey in West Virginia, the numbers of tree holes were recorded in 994 identified trees in single transects at 12 sites. For the most productive species of tree, the proportions with tree holes were as follows: *Acer rubrum*, 0.07 (n = 177); *Acer saccharum*, 0.09 (n = 159); *Betula lenta*, 0.07 (n = 43); *Hamamelis virginiana*, 0.13 (n = 24); *Amelanchier arborea*, 0.60 (n = 15); *Ulmus rubra*, 0.25 (n = 12); and *Quercus rubra*, 0.10 (n = 10) (Nasci *et al.*, 2000).

Studies in woodland north of Peoria Heights, Illinois, revealed the productivity of tree holes. During the period 1978–1981, occupied tree holes in 50 trees of ten species yielded a total of 22,021 Triseriatus Group larvae or pupae. The highest mean numbers per tree were: *Fraxinus americana*, 1841; *Ulmus americana*, 1011; and *Quercus rubra*, 533. Oaks (*Quercus*) accounted for 72% of trees with tree holes containing water. *Quercus rubra* provided 46% of the trees sampled, and yielded 55.7% of the mosquitoes tested. Twenty-seven isolates of LACV were obtained from 88 pools of adults reared from the 22,021 aquatic stages collected (MIR = 1.2) (Clark *et al.*, 1983).

Females of *Oc. triseriatus* also oviposit in artificial containers, notably disused car tyres. Vertically infected eggs laid in such tyres could be transported over considerable distances (Thompson, 1983b; Nasci *et al.*, 2000).

(b) Development

Ochlerotatus triseriatus is geographically polymorphic for its stage of dormancy, which may be the 'egg' stage or the fourth-instar larva, an adaptation to the differences in severity of winter conditions at different latitudes. Northern populations, between 42° N and 50° N, are univoltine or partly bivoltine, and overwinter exclusively as 'eggs' – in fact, as pharate, first-instar larvae within the eggshell. Southern populations are multivoltine and overwinter as 'eggs' or as fourth-instar larvae; the tendency for 'egg' diapause diminishes at lower latitudes. Diapausing 'eggs' can survive through winters in frozen tree holes; apparently, diapausing larvae cannot (Holzapfel and Bradshaw, 1981; Sims, 1982).

Both egg diapause and larval diapause are induced by exposure of the respective stage to short day length, supplemented by cool or low temperature; in the case of 'eggs', both embryo and pharate first-instar larva are photosensitive. The critical day length and temperature threshold needed to induce egg diapause can be longer and higher, respectively, than are required to induce larval diapause; therefore, egg diapause is induced earlier in the summer than larval diapause. Termination of diapause is stimulated by low temperature supplemented by long day length (Kappus and Venard, 1967; Wright and Venard, 1971; Shroyer and Craig, 1980, 1983; Holzapfel and Bradshaw, 1981).

Temperature also affects metamorphosis. In southern Wisconsin, for the generation that has overwintered and resumed development, a relatively high tree hole water temperature is necessary for the start of pupation. Usually this temperature threshold delays the start of pupation until at least the last week of May. Adult males emerge before females. In 1976 and 1977, at a site in Iowa County (Hanson's Farm), records of the sex ratio of emerging *Oc. triseriatus* showed a bias towards male emergence during the early part of the season and towards female emergence during the later part. In 1976 the switch of sex ratio occurred in mid- to late July. In 1977, because of unusually warm weather, the switch occurred in late June. In that year tree hole water temperatures had been 4.2°C higher than normal, permitting earlier pupation, and emergence had started 3 weeks earlier than in 1976. On average in the 2 years, males reached cumulative 50% emergence 32 days before the females.

The tendency for females to emerge later delays the laying of any substantial number of eggs at least until the last week in June. The late start of oviposition leaves only a narrow time gate for eggs

laid by the overwintered females to hatch. Only eggs laid up to mid-July, a small proportion of the season's total, would be expected to hatch soon after oviposition; the remainder enter diapause. Scholl and DeFoliart (1978) concluded that in years with normal weather patterns only a partial second generation develops, producing an estimated mean of 1.2 generations per year. In 1975, at another site in Iowa County (Kaeser's Farm), the period of high oviposition activity by Oc. *triseriatus* lasted for 10 weeks, from the week ending 3 July to the week ending 4 September (Scholl and DeFoliart, 1977).

Different findings were reported for a population of Oc. *triseriatus* at 42° 45' N in southern Ontario. When based on monthly sampling of tree-hole populations in the years 2003 to 2005, the timing of larval development suggested that at that latitude Oc. *triseriatus* was univoltine, with an unsuccessful second generation. In contrast, weekly sampling of six tree holes in American beech during the ice-free season of 2006 was said to indicate three generations and an unsuccessful fourth generation. The weekly sampling regime was considered to produce more valid data (Williams *et al.*, 2007). The evidence for a multivoltine life cycle was presented without discussion of the characteristics of development in the egg and larval stages of Oc. *triseriatus*, or of its diapause physiology. A gradual release from diapause is characteristic of Oc. *triseriatus*, followed by a prolonged phase of hatching so that first instar larvae appear over a prolonged period. In an investigation in which diapausing eggs of Oc. *triseriatus* eggs were kept outdoors under field conditions at Madison, Wisconsin (c. 43° 5' N) and sampled at 5-week intervals, some eggs remained in diapause for up to 27 weeks after diapause induction (Figure 45.26), and most post-diapause eggs needed a number of exposures to hatching stimuli (McGaw *et al.*, 1998).

(c) Adult biology

Adult female Oc. *triseriatus* feed in woodlands – at ground level, at intermediate heights and in the canopy. About one-half of the females caught biting were taken at ground level. Biting activity occurs from sunrise to sunset, but the proportion biting at ground level was lower in the early morning and late afternoon (Scholl *et al.*, 1979a; Novak *et al.*, 1981). *Ochlerotatus triseriatus* is an opportunistic feeder; when woodland amphibians, reptiles, mammals and domestic chickens were exposed as bait in a natural habitat, blood meals were taken on all classes. However, forage ratios have not been measured. Serological typing of blood meals of females collected at ground level in woodlands in areas where LACV was endemic showed Oc. *triseriatus* to have fed predominantly on mammals, and substantially on white-tailed deer and sciurid rodents (Table 45.21). Even taking into account the probable feeding of Oc. *triseriatus* on squirrels in the canopy, which might not have been measured in these studies, Burkot and DeFoliart (1982) estimated that the sciurid rodents were the source of less than one-half of Oc. *triseriatus* blood meals. The red fox was not among the species identified as blood sources in those investigations, but when exposed as bait at locations in Wisconsin it was attacked by Oc. *triseriatus* (Wright and DeFoliart, 1970).

Some aspects of the biology of Oc. *triseriatus* are difficult to examine or to measure directly on wild mosquitoes, e.g. feeding success or duration of gonotrophic cycles, so most investigators have worked either in the laboratory or in the field with laboratory-reared mosquitoes. All three approaches have limitations and may produce erroneous results, but the findings obtained in strictly natural situations are more likely to be realistic. The survival rates of adult Oc. *triseriatus* have been measured in mark–release–recapture experiments. The daily survival rate of wild-caught females was 0.87 (Sinsko and Craig, 1979), while those of females from three colonies were 0.80, 0.94 and 0.95 (Beier *et al.*, 1982; Haramis and Foster, 1983 Walker *et al.*, 1987).

(d) Vector status

Ochlerotatus triseriatus has been identified as the main vector of La Crosse virus on the following

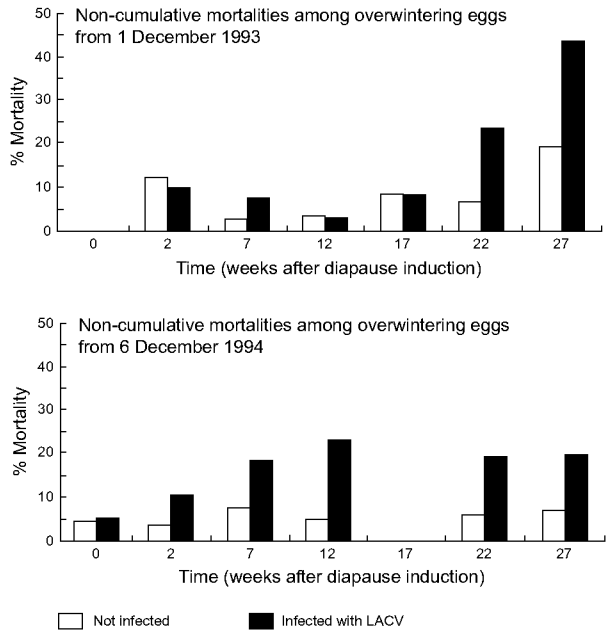


Figure 45.26 Mortalities in overwintering eggs of *Ochlerotatus triseriatus* that were either vertically infected with La Crosse virus or not infected. (After McGaw *et al.*, 1998.) Egg viability was measured at 5-week intervals after the start of diapause induction. Diapause was induced (at time 0) by placing recently embryonated eggs at 21 °C and subjecting them to a shortened day length (LD (light:dark) 10:14) for 2 weeks, when all were in diapause. Eggs from an F_3 generation from the wild were used in the very cold winter of 1993–1994 (top), and from an F_7 generation during the less cold winter of 1994–1995 (bottom). Two weeks after the start of diapause induction, eggs were sent to Madison, Wisconsin (c. 43° 05' N), and placed outdoors where the mean daily temperature remained subzero until late March. At 5-week intervals thereafter, randomly selected eggs were withdrawn (presumably transferred to non-diapause conditions) and tested for the following: viability; hatching rate of surviving eggs in response to 1–4 immersions in hatching medium over a period of time; and filial infection rate. In the 1993–1994 winter (top), the eggs began to terminate diapause in response to a photoperiod in excess of LD 13:11 between mid-March and the end of April; at week 27, in the final week of May, some eggs were still in diapause.

grounds. (i) LACV has been isolated from wild-caught Oc. *triseriatus* in a number of locations where the virus is endemic. (ii) Its host preference is for mammals, including the known amplifying hosts. (iii) The adults live in the same habitats as the amplifying hosts of LACV, and in close association with them. (iv) The seasonal period of its host-feeding activity coincides with that of disease incidence. (v) The vectorial competence of Oc. *triseriatus* has been demonstrated in the laboratory. Field studies have shown Oc. *triseriatus* to be the main vector of La Crosse virus over the whole range of that virus. In many locations, LACV is vertically transmitted in populations of Oc. *triseriatus*.

45.5.5 Biology and vector status of other mosquito species

All four species of the Triseriatus Group are susceptible to oral infection by LACV in the laboratory; in fact, the proportion of females permitting dissemination of the virus was lowest in Oc. *triseriatus*. The capability of transmitting LACV to mice ranged from very low for Oc. *hendersoni* to highest for Oc. *zoosophus* (Table 45.22) (Paulson *et al.*, 1989). All four species can transmit LACV, but only Oc. *triseriatus* is considered a natural vector.

Ochlerotatus canadensis occurs widely in forested areas of North America, where the aquatic stages develop in ground pools and the adult females are

Table 45.21 The blood-meal sources of engorged mosquitoes (*Ochlerotatus triseriatus*) captured in woods in areas of northern Indiana and south-western Wisconsin where La Crosse virus was endemic. (From the data of Nasci (1982) for Indiana; and Burkot and DeFoliart (1982) for Wisconsin.) Battery-powered aspirators were held by operators working at ground level. The results for engorged *Aedimorphus vexans* are included for comparison.

Mosquito species	*White-tailed deer* (%)	*Chipmunk + tree squirrel* (%)	*Other mammals* (%)	*Birds* (%)	n
Indiana					
Oc. triseriatus	43.8	41.7	8.3	6.2	48
Wisconsin					
Oc. triseriatus	64.8	24.0	10.7	0.5	196
Am. vexans	93.7	0.8	4.4	0.8 *	366

*, The midgut contents of some engorged *Am. vexans* included reptile blood.

opportunistic feeders (Volume 2, Sections 39.3.1.a, 39.3.4). Wild-caught and F_1 females of *Oc. canadensis* that were orally infected transmitted LACV to mice, but somewhat less effectively than *Oc. triseriatus* (Watts *et al.*, 1973). Among 72 females of *Oc. canadensis* that had been reared from wild-caught larvae and that fed on viraemic mice, 75% became infected (n = 72). Among ten chipmunks that were bitten by either one or two infected *Oc. canadensis*, three became infected (Berry *et al.*, 1986). Adult *Oc. canadensis* infected with LACV have been collected in at least two states. (i) In Ohio, from 1965 to 1981, infected females were recorded in 64 counties, and in two representative counties MIRs of 3.6 (n = 1659) and 6.9 (n = 1162) were obtained (Berry *et al.*, 1986). (ii) In West Virginia, LACV-infected females of both *Oc. canadensis* and *Oc. triseriatus* were captured in landing catches at two sites. At one site the respective MIRs were 1.6 (n = 600) and 12.8 (n = 156), and at the other 2.4 (n = 820) and 4.3 (n = 230). Nasci *et al.* (2000) concluded that, given these infection rates and the large population densities that *Oc. canadensis* can attain, it may be an important subsidiary vector in areas where LACV transmission is maintained by *Oc. triseriatus*.

The South-east Asian tree-hole mosquito *St. albopicta* has invaded and progressively expanded its range in the USA, where its ability to exploit artificial containers brings it into close association with human populations. It is an ecological generalist capable of rapid genetic modification and, where humans are present, of rapid colonization of new habitats (Hawley, 1988, review). At a focus of LACV transmission in Illinois (at 40° 41' N), a population of *St. albopicta* survived as dormant eggs through the mild winter of 1997–1998 (Swanson *et al.*, 2000). In Tennessee, LACV was isolated from adult *St. albopicta* reared from eggs laid in oviposition traps, showing a capability for vertical transmission (Gerhardt *et al.*, 2001). Orally infected females of *St. albopicta* (the F_2 or F_3 offspring of wild-caught parents) transmitted LACV to suckling mice at rates equal to or greater than those of *Oc. triseriatus* (Grimstad *et al.*, 1989). Female *St. albopicta* became infected through feeding on viraemic chipmunks, and infected females could transmit the virus to chipmunks (Cully *et al.*, 1992). In a blinded cohort study of 38 children with suspected CNS infection conducted in eastern Tennessee, 15 children were later shown to be infected with LACV and 23 not infected. Mosquito larvae and adults were collected at all 'primary exposure sites' of the 38 children, and the numbers recorded. The mean numbers of larvae + adults were as follows. (i) sites visited by LACV-infected children: *St. albopicta* 17.36; *Oc. triseriatus* 1.71; (ii) sites visited by non-infected children: *St. albopicta* 5.43; *Oc. triseriatus* 10.09. Erwin *et al* (2002) concluded that *St. albopicta* may be involved in the

Table 45.22 Rates of infection with La Crosse virus of *Ochlerotatus* species (Triseriatus Group) after oral intake, and rates of transmission of LACV to suckling mice. (From the data of Paulson *et al.*, 1989.)

			Oral transmission	
Species	*No.*	*Disseminated infection* (%)	*All females* (%)	*Disseminated females* (%)
Oc. *triseriatus*	171	67	37	56
Oc. *hendersoni*	135	84	7	9
Oc. *brelandi*	55	89	27	31
Oc. *zoosophus*	65	99	85	86

Oc. *triseriatus* and Oc. *hendersoni* were represented by two strains each, and the other two species by one strain each. The mosquitoes imbibed defibrinated rabbit blood containing $10^{6.1}$ – $10^{7.2}$ $TCID_{50}$ (50% tissue-culture-infective dose) ml^{-1} LACV from an artificial-membrane feeder. Disseminated infection was determined from head-squash preparations. Rates of oral transmission were determined by the effects of feeding on suckling mice; the results were calculated for the total test population and separately for females showing a disseminated infection.

emergence of La Crosse encephalitis in eastern Tennessee. Vertical transmission of LACV by *St. albopicta* was demonstrated in the laboratory, but the maternal infection rate was lower and the filial infection rate much lower than in Oc. *triseriatus* (Hughes *et al.*, 2006).

45.5.6 Transmission cycles

In the cooler part of its range, the annual transmission cycle of LACV has two phases: (i) a dormant phase, during the winter, when the virus survives in diapausing, vertically infected eggs of *Ochlerotatus triseriatus*; and (ii) an amplification phase, during which it is transmitted between adult Oc. *triseriatus* and its mammal hosts. The most detailed investigations into LACV transmission have been undertaken in the part of Wisconsin that is endemic for LACV, which corresponds roughly with the unglaciated south-western region of the state (Lisitza *et al.*, 1977). Most study sites were in La Crosse County (43° 43' N to 44° 05' N) or Iowa County (42° 48' N to 43° 12' N).

(a) Habitat

In Iowa County, studies were undertaken at six sites (named in Table 45.23) during the 2 years 1974 and 1975, or during 1974 or 1975 only, when Oc. *triseriatus* larvae that had hatched from overwintered eggs were collected, pooled and tested for vertical infection with LACV. The MIRs, which ranged from 0 to 5.9, were generally lower than those recorded in La Crosse County. Both tree holes and Oc. *triseriatus* larvae were abundant at the two sites at Burkholder (Iowa County), which were on opposing south-facing (Burkholder S) and north-facing (Burkholder N) slopes. The south-facing slope was the drier of the two, and there both ground cover and chipmunks were relatively scarce. The significantly higher prevalence of LACV at the Burkholder N site was consistent with an earlier finding (by Gauld *et al.*, 1974) that LACV activity is greater in areas where the vegetation is dense and chipmunks occur at higher densities. The Hanson and Hanson B sites occupied adjacent south-facing sites, with only a narrow dirt road separating them, yet they differed significantly in LACV prevalence ($p > 0.01$) (Table 45.23). Lisitza *et al.* (1977) surmised that this was owing to differences in their vertebrate populations: the Hanson B site was used by deer for bedding down, and appeared to have fewer chipmunks than Hanson, where virus prevalence was higher.

Table 45.23 Prevalence of La Crosse virus in larvae of *Ochlerotatus triseriatus* that had developed from overwintered eggs. The larvae were found in tree holes at six sites in Iowa County, south-western Wisconsin, during the years 1974 and/or 1975. (From the data of Lisitza *et al.*, 1977.)

	MIR/1000		
Site	*1974*	*1975*	*Total larvae tested*
Hanson	3.4	2.9	7350
Hanson B	–	0.2	9693
Davis	5.9	2.9	2917
Burkholder S	0	0.3	5775
Burkholder N	–	1.3	4474
Kaser	1.4	–	2187

Larvae were collected periodically between 14 May to 14 June 1974 and 15 May to 6 June 1975. To ensure that the larvae had hatched from overwintered eggs, no collections were made after the first pupae were found.
MIR, minimum infection rate.
–, Not sampled.

(b) Overwintering of LACV

In Wisconsin, winters are hard, and Oc. *triseriatus* overwinters in the egg stage as pharate, first-instar larvae. To examine the effects of winter conditions on eggs infected or not infected with LACV, some females of Oc. *triseriatus* were parenterally infected with the virus, and batches of infected and uninfected eggs were induced to enter diapause and then placed outdoors for periods of up to 27 weeks. At intervals of 5 weeks after diapause induction, batches of eggs were removed and tested for viability, readiness to hatch in response to repeated exposures to hatching stimuli, and vertical transmission rate. The study was undertaken during two consecutive winters, the temperatures being markedly lower during the first winter. By week 22 after diapause induction, the eggs were beginning to come out of diapause.

During the first winter the death rate was low in the diapausing eggs (weeks 2–17), but high in the post-diapause eggs (Figure 45.26). The overall survival rates at the end of the first winter were: infected eggs, 56.4%; uninfected eggs, 80.8%. During the second winter the uninfected eggs suffered little mortality, but the infected eggs, both diapausing and post-diapause, suffered a relatively greater mortality. The overall survival rates at the end of the second winter were: infected eggs, 80.8%; uninfected eggs, 92.7%. During the first winter, the vertical transmission rate declined from 0.62–0.63 at the outset to 0.53 at the end, possibly owing to the higher death rate among infected eggs, but during the second winter it declined only from 66.5% to 64.9%. Measurements of viral transcription and replication within the eggs showed mRNA and rRNA to be present during all time periods, suggesting that some replication occurred during diapause (McGaw *et al.*, 1998).

During the spring and early summer of 1974, in forest in La Crosse County, tree holes were sampled for Oc. *triseriatus* larvae that had hatched from overwintering eggs. The larvae were reared to the adult stage and then individually tested for vertical infection with La Crosse virus. Of 103 water-filled tree holes, 64 contained larvae (n = 1698). Fifty-five of those tree holes yielded only uninfected larvae (n = 1188), while nine yielded larvae positive for LACV (7♂♂ and 3♀♀) (n = 510). The MIR for larvae from the infected tree holes was 19.6, while that for the whole larval population was 5.9. In one study area, all tree holes were sealed with mesh screen on 14 June, before adult emergence, to prevent the escape of adults and re-entry of gravid females. Infected adults emerged within those tree holes up to the final collection on 12 August, which suggests that some vertically infected individuals of the overwintering generation contribute virus to the transmission cycle in mid-summer (Beaty and Thompson, 1975).

(c) Amplification during summer transmission cycles

With LACV infections, as with those of most or all other mosquito-borne arboviruses that may be vertically transmitted, the prevalence rate of infection among the vertically infected, egg-laying females of one generation is always higher than that among the newly emerged adult females of the next. Not all of the progeny of a female with infected germ cells are vertically infected, consequently the filial infection rate among the progeny of any infected female is <1.0, and the vertical transmission rate among the progeny of the cohort of infected

females is <1.0 (Section 44.6.5). The effects of this fall are most obvious when arboviruses survive through periods of adverse climatic conditions in vertically infected, diapausing eggs. The lower prevalence rate in the generation that emerges from diapause needs to be compensated for by amplification during the following phase of horizontal transmission.

From successfully overwintered, vertically infected eggs of *Oc. triseriatus*, LACV can be transmitted, amplified and perpetuated in two ways. (i) A vertically infected adult female can transmit LACV to an amplifying host by bite, and one or more females that feed on that host when it is viraemic can become infected and lay vertically infected eggs at the end of the second gonotrophic cycle. (ii) A vertically infected adult male can, during mating, pass the virus to an uninfected female, which later transmits it by bite to one or more amplifying hosts.

In the laboratory, the extent of horizontal transmission of LACV was enhanced by venereal transmission when uninfected females of *Oc. triseriatus* mated with vertically infected males a number of days after engorging. When females fed on chipmunks with anti-LACV neutralizing antibodies 5–11 days before becoming venereally infected, any such enhancement was diminished (Section 44.5.2.c).

For amplification to occur, an amplifying host must become infected by the bite of a vertically infected female, a number of uninfected females must feed on that host while it is viraemic, become infected, and survive long enough to lay batches of vertically infected eggs. The extent of amplification is constrained by the deaths of some females during the period between feeding and laying vertically infected eggs. When adult female *Oc. triseriatus* reared from wild-caught larvae were orally infected with LACV, and the extent of vertical transmission to their offspring was measured, the vertical transmission rates were zero for progeny from the first gonotrophic cycle, 43% for progeny from the second, and 58% for progeny from the third (total $n = 1022$). If the daily survival rate were 0.87 (as determined by Sinsko and Craig, 1979), only about 10% of orally infected females would survive to lay vertically infected eggs. In fact, among 558 wild females caught while biting, only 8% were biparous, and so capable of depositing vertically infected eggs if themselves horizontally infected (Miller *et al.*, 1979). In mark–release–recapture experiments, the mean age at recapture of females that were uniparous was 15–16 days (Scholl *et al.*, 1979b).

Apparent evidence of amplification was obtained in two field investigations, when higher MIRs were demonstrated in wild-caught adult *Oc. triseriatus* than in larvae and pupae collected from tree holes or in adults reared from those aquatic stages. First, we note a 4-year study in woodland in Illinois, in which collections from 50 tree holes yielded a total of 22,021 *Oc. triseriatus* larvae or pupae. When reared to the adult stage, tests for LACV showed an overall MIR of 1.2 (by sex: ♂♂, 1.04; ♀♀, 1.48). During one year, when two humans were used as bait in catches during 2-day exposures at the beginning of each month, the overall MIR for the landing-biting catch was 9.9, whereas the MIR for females collected at the ten tree holes nearest the collectors was only 5.4. Analysis of landing-catch data for three summer months gave the following results: July, MIR = 12.6 ($n = 872$); August, MIR = 10.5 ($n = 1800$); September, MIR, 6.5 ($n = 2775$). Using additional data, it was shown that the number of infected females flying in July was less than half that in August, while the number fell only slightly in September. Although the MIR decreased as the summer progressed, the risk of an infected bite was highest in August and September. The investigators tentatively ascribed the increase in numbers of infected females to amplification (Clark *et al.*, 1983, 1985).

In an investigation at 12 sites in West Virginia, eggs collected from ovitraps were reared to the adult stage, sorted to species and tested for LACV; adult females captured in landing-biting catches were similarly tested. LACV was isolated from adults reared from eggs collected at all 12 sites; the range of mean MIRs was 0.4–7.5 ($n = 73,479$). LACV was isolated from pools of *Oc. triseriatus* caught in landing catches at only five of the 12 sites, where the range of MIRs was 4.3–21.7 ($n = 1055$) (Nasci *et al.*, 2000).

(d) Modelling transmission

The principal concern of modellers has been to find how LACV might be sufficiently amplified during the phase of horizontal transmission to balance the losses associated with vertical transmission. Under particular experimental conditions, Miller *et al.* (1977) had shown a loss of 30% in filial infection rate at each vertical transmission of the virus from one generation to the next. They postulated that, in the absence of amplification or of any selective advantage or disadvantage deriving from infection, the maternal infection rate (m) among the adult F_1 offspring of vertically infected females in an overwintered population is expressed by

$$m = a\,b^n \qquad (45.1)$$

where a is the proportion of overwintered females vertically infected with LACV, b is the vertical-transmission rate from the overwintered females to their offspring, and n is the number of generations of Oc. *triseriatus* during the year. The amount of amplification (A) needed during the summer to maintain the maternal infection rate from one year to the next is given by

$$A = a(1-b)^n \qquad (45.2)$$

Assuming that there were two generations per year and the vertical-transmission rate was 70%, by the end of the year the number of infected eggs would be about 50% of the number needed to maintain the prevalence of overwintering virus. Under such conditions, the virus might persist for 4 years or longer in the absence of amplification in mammalian hosts. As noted above, at the latitude of Wisconsin, populations of Oc. *triseriatus* pass through only one generation plus a 'partial second' per year.

Burkot and DeFoliart (1982) developed a preliminary deterministic model to find whether sufficient amplification of LACV occurs in wild vertebrate hosts to balance the supposed 30% loss during vertical transmission. Into the (undescribed) model, they fed quantitative data from their study site in Iowa County. Commenting that fewer than 50% of blood meals of Oc. *triseriatus*, and possibly as few as 24%, are taken on amplifying hosts, they concluded that some 25–50% of the vector population derives its blood meals from non-amplifying hosts, and that 25–50% of infective bites are wasted on non-amplifying hosts. In a worst-case scenario, with only 25% of vertically infected vectors delivering virus to amplifying hosts, and only 25% of susceptible vectors feeding on amplifying hosts, transmission of LACV would be 16 times less efficient than if the vectors fed only on amplifying hosts. They concluded that either their estimates of horizontal transmission had been too low to provide the necessary amplification, or an additional form of horizontal transmission was involved. Venereal transmission may contribute to amplification of LACV (Section 44.5.2.c). Recoveries through a period of 38 days during which marked adult Oc. *triseriatus* were released into woodland showed the probability of survival through one day (p) to be 0.81 for males and 0.80 for females. Beier *et al.* (1982) commented that, if wild males live as long as wild females, that might enhance the extent of venereal transmission.

From an analysis of time versus titre profiles of viraemia in chipmunks, Patrican *et al.* (1985b) concluded that each infective bite delivered by vertically infected females to non-immune chipmunks yields an average of 0.9 days of high viraemia and 0.7 days of a lower viraemia. They estimated that 90% of mosquitoes that fed on chipmunks during a period of high viraemia would transmit LACV upon refeeding, but that only 25% of mosquitoes that fed on chipmunks during a period of low viraemia would transmit. These authors developed an algebraic expression for the number (a) of Oc. *triseriatus* that, having become orally infected by feeding on a viraemic chipmunk on a certain day, survive and transmit LACV to immune chipmunks at a later blood meal. Thus,

$$a = b\,(cd + ef)\,(g)^h \qquad (45.3)$$

where: b is the daily biting rate (10) on a chipmunk; c is the number of days (0.9) of high-titre viraemia; d is the capability of transmission (0.9) by females that become orally infected during the period of high-titre viraemia; e is the number of days (0.7) of low-titre viraemia; f is the capability of transmission (0.25) by females infected during the period of low-

titre viraemia; g is the daily survival rate (0.95); and h is the extrinsic incubation period in days (10). Accepting the authors' measured or assumed values (in parentheses), the value of a = 5.89. If a daily survival rate of 0.87 (as determined by Sinsko and Craig, 1979) is used, the value of a falls to 2.7.

Amplification of LACV undoubtedly occurs in certain of its vertebrate hosts, but the extent of the amplification has not been measured. DeFoliart (1983) identified four principal factors that lessen the extent of amplification: (i) wastage of infective bites on non-amplifying species; (ii) wastage of infective bites on immune amplifying hosts; (iii) the low proportion of vector females that, at the perceived host and vector population densities, become orally infected through feeding on viraemic amplifying hosts; and (iv) the deaths of infected vectors that occur between the infecting blood meal and the second post-infection oviposition.

An attempt was made to calculate the effects of these factors on the amplification of LACV in a theoretical population of Oc. *triseriatus* that occupied 100 ha of woodland in an area endemic for LACV. It was given characteristics similar to those of a much-studied population at Hanson's Farm, Iowa County. Females were considered to emerge from tree holes at the rate of 150 per day for 100 days, and the prevalence rate of LACV among females emerging from overwintered eggs was taken to be 0.05. Females that became infected orally at the start of one gonotrophic cycle first laid vertically infected eggs at the end of the following gonotrophic cycle. The density of the sciurid amplifying hosts was taken to be 30 ha^{-1}, of which 30% were treated as immune. Some 75% of blood meals were taken on non-amplifying hosts. Calculations showed that, in a population with these characteristics, the extent of LACV amplification during phases of horizontal transmission would fail to compensate for the reductions in prevalence rate that happen during phases of vertical transmission (Table 45.24). DeFoliart (1983) noted, however,

Table 45.24 Estimated amplification of La Crosse virus resulting from bites on sciurid rodents delivered by the overwintering, vertically infected female component of a population of *Ochlerotatus triseriatus*. (From DeFoliart, 1983.) The numerals in columns 5–9 of the table are calculated values for a 10 ha area of forest.

1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Oviposition	*Blood meal*	*At days post-eclosion* (n)	*Mean survival* (%)	*Bites delivered* (n)	*Bites on amplifying hosts* (n)	*Viraemias produced* (n)	*Orally infected* ♀♀ (n)	*Infected egg masses* (n)
(Mating)	1st	4	57.3	429.7	107.4	75.2	15.0	1.4
1st	2nd	12	18.8	141.0	35.3	24.7	4.9	0.5
2nd	3rd	21	5.4	40.3	10.1	7.1	1.4	0.1
3rd	4th	30	1.5	11.5	2.9	2.0	0.4	0
Totals (columns 5–9)				622.5	155.7	109.0	21.7*	2.0
Total egg masses laid				192.8**				

Assumptions: In a 100 ha forest, a mean of 300,000 Oc. *triseriatus* (150,000 ♂♂ and 150,000 ♀♀) emerge each season. The females emerge at the rate of 1500 per day for 100 days. The prevalence rate for infection with LACV in adult females that develop from overwintering eggs is 0.05. The probability of survival through one day (p) = 0.87. The resident amplifying-host population totals 3000, of which 30% are immune. Only 25% of bites are on amplifying hosts.

Col. 5: n = 750 × col. 4 (%) (15,000 × 0.05 = 750). Col. 6: n = 0.25 × col. 5. Col. 7: n = 0.7 × col. 6 (70% immune). Col. 8: n = 0.2 × col. 7 (20% infection rate). Col. 9: n = 0.094 × col. 8 (p = 0.87, so 9.4% of orally infected females survive the 17 days to the second post-infection oviposition).

*, These females have infected salivary glands before the first post-infection blood meal, and they survive to deliver 9.7 infective bites. Their ovaries become infected before the second post-infection oviposition, and they survive to deposit 2.0 infected egg masses.

**, With a vertical transmission rate of 0.7, only 135.0 of these egg masses are infected. For 192.8 infected egg masses to be produced, a total of 275.4 egg masses is required. Therefore 82.6 infected egg masses must be produced through amplification during a horizontal transmission cycle to maintain LACV at a steady prevalence rate.

that venereal transmission might partially compensate for the deficit in numbers of vertically infected eggs.

45.5.7 Summary

La Crosse virus is perpetuated in transmission cycles that involve sciurid rodents as amplifying hosts and *Oc. triseriatus* as the main vector. In cold temperate parts of the range of LACV, the annual transmission cycle has phases of vertical and horizontal transmission. LACV overwinters in dormant, vertically infected eggs of *Oc. triseriatus*, and during the following summer it is amplified in an enzootic, horizontal transmission cycle. Probably, deer are dead-end hosts that diminish the circulation of LACV. Humans also are dead-end hosts. Overwintering in vertically infected *Oc. triseriatus* eggs is an essential stage in the seasonal cycle of LACV, but owing to the inevitable diminution of the virus population during the vertical transmission phase it must, for its long-term perpetuation, be amplified to an equal extent during the phase of horizontal transmission. In a mathematical analysis of theoretical host and vector populations that were given characteristics of populations in a well-studied endemic area, the estimated extent of amplification during horizontal transmission was insufficient to make up for the accrued deficits. The introduction of venereal transmission into the analysis might modify that result.

45.6 TRANSMISSION OF RIFT VALLEY FEVER VIRUS

Rift Valley fever virus (RVFV), type species of the genus *Phlebovirus* (family *Bunyaviridae*), infects a wide variety of African mammals and is highly pathogenic in domesticated ruminants. Sudden and severe epizootics of Rift Valley fever, sometimes accompanied by epidemic human disease, have occurred spasmodically throughout sub-Saharan Africa, and more recently in the Sudan, Egypt and the Arabian peninsula. They appear as outbreaks of acute, haemorrhagic disease in domesticated sheep, goats or cattle, with high mortality among newborn animals. Abortion in pregnant females causes much economic loss. Humans who are in close contact with diseased livestock can become infected, and in some countries RVFV has caused many human deaths. The pathology of the disease is described in Section 44.10.3.c.

45.6.1 Introduction

(a) First description of RVFV

In 1930, an outbreak of disease on a farm in the Rift Valley caused the death of thousands of newly born lambs. The affected farm was situated in open bush country in the region of Naivasha, Kenya, where in 1930 the rainfall was 80% above average. At the time of death, the lambs were 3–7 days old, most about 3 days old. Coincident with the appearance of disease in lambs, there was a marked rise in the death rate of ewes, and the number of abortions also increased. Ewes and lambs that were moved to a higher adjoining farm at the edge of the Rift Valley, at an altitude of 2000–2500 m, and that survived for more than 5–6 days after arrival, remained healthy, while on the lower farm the disease continued for several months. A high death rate was noted among two rodents, *Arvicanthis nairobae* and *Rattus rattus*, on the lower farm. Investigations by four veterinary research scientists during the course of the outbreak revealed important aspects of the epidemiology of the disease. During that period, all four research workers became infected.

To isolate the infectious agent, blood taken from a naturally infected lamb was inoculated intravenously into a sheep. After two passages through sheep, purification procedures yielded a virus which, when inoculated into non-immune sheep or cattle, produced characteristic symptoms including cytological changes and lesions within the liver. The viral disease was named Rift Valley fever (RVF). From the earliest experiments, experimentally infected sheep were housed with susceptible sheep, but infection through contact never occurred. To test whether the virus was transmitted by mosquitoes, batches of sheep were kept housed or placed in pasture, and were protected by mosquito netting or left unprotected. With one exception, only sheep that were free in pasture at night became infected. Inoculation of homogenates of many hundreds of *Cx. pipiens* or *Cx. quinquefasciatus* into sheep caused no disease, but a lamb inoculated with a homogenate of five '*Taeniorhynchus brevipalpis*' showed progressive leucocyte changes characteristic of RVF, and the lamb became immune (Daubney and Hudson, 1931). However, '*Taeniorhynchus brevipalpis*' is not a recognizable name; nor is '*Mansonia brevipalpis*'.

(b) Initial characteristics of RVF in domesticated animals

Among domesticated animals, Rift Valley fever is a disease of sheep, goats, cattle, buffalo, camels,

horses and donkeys. Usually, viraemia is demonstrable at the onset of fever, and can persist for up to a week. Maximum recorded virus titres (as mouse LD_{50} ml^{-1}) were $10^{10.1}$ in lambs, $10^{8.2}$ in kids and $10^{7.5}$ in calves. Somewhat lower maximum titres were recorded from adult animals (Swanepoel, 1998; Boden, 2001). In duplicated experiments in which lambs were inoculated with 10^5 PFU of RVFV, the peak viraemias in 3-day-old lambs were $10^{6.2}$ and $10^{7.0}$, and in 6–8-week-old lambs they were $10^{5.0}$ and $10^{5.3}$ PFU ml^{-1}. Viraemias were first detected after about 24 h, were highest between 28 h and 52 h, and were barely detectable by 72 h post-inoculation. In 13 out of 14 experiments, the median viral titre recovered from engorged *Cx. pipiens* was $>10^{2.0}$ PFU (Turell *et al.*, 1984b). Most domesticated ruminants are amplifying hosts.

(c) *Outline history of Rift Valley fever*

Rift Valley fever had 'undoubtedly existed in the Rift Valley for some years' before its recognition in Kenya in 1930, in the view of Daubney and Hudson (1931). An illness called 'dioundé' by indigenous people in the regions of Ségou and Macina, in what is now Mali, had characteristics of Rift Valley fever, and serological tests confirmed that identification (Stéfanopoulo, 1933; Curasson, 1934). Limited surveys revealed seropositivity for RVFV among indigenous peoples in Uganda, southern Sudan, the Republic of Congo and Gabon (Findlay *et al.*, 1936).

During the four decades after the designation of Rift Valley fever, RVFV was isolated and outbreaks of the disease were reported from eastern Africa (as far north as Kenya) and southern Africa. In 1944, RVFV was isolated from mosquitoes in Semliki Forest in western Uganda (Smithburn *et al.*, 1948). Epidemics occurred in South Africa in six of the years from 1950 to 1976, in Namibia in 1955 and 1974, and in Kenya in three of the years from 1968 to 1979. Losses could be very heavy. During an epidemic in South Africa in 1950–1951, 100,000 sheep died and 500,000 ewes aborted (Meegan and Bailey, 1989; Bouloy, 2001). No human deaths were attributed to RVF until the 1975 South African epidemic, when three fatalities were recorded among a small number of human cases (Van Velden *et al.*, 1977).

Zinga virus was first isolated during 1969 in the Central African Republic, from *Mansonia africana* and *Neomelaniconion palpale*-group mosquitoes, and later it was isolated from the blood of a febrile human (Digoutte *et al.*, 1974a,b). Subsequently, Zinga virus was shown to be identical with RVFV (Meegan *et al.*, 1983). A search for arboviruses in Ibadan, Nigeria, in 1967–1970, identified RVFV in *Culicoides* sp. (Ceratopogonidae) and *Cx. antennatus* (Lee, 1979). During 1984 and 1985, in the Central African Republic, RVFV was isolated from five human serum samples, three being from patients who died from haemorrhagic disease (Meunier *et al.*, 1988).

During the 1970s, outbreaks of RVF occurred in the Sudan and Egypt. The first recorded outbreaks north of Kenya were an epidemic among livestock 200 km south of Khartoum in 1973, and a smaller outbreak there in 1976 (Eisa *et al.*, 1980; Saleh *et al.*, 1981; Meegan and Bailey, 1989). Seropositivity among humans from different regions of the Sudan was reported by Saleh *et al.* (1981). The first indication of the virus reaching Egypt was in seropositive samples obtained from sheep, cow and buffalo at Kena in southern Egypt in February 1977. In March–April 1977, seropositivity was detected in animals in the northern part of the country, and in early October 1977 an outbreak of RVF in humans started in Sharqīyah Province, north of Cairo (WHO, 1977, 1979). An epidemic of RVF developed in the Nile Valley and Nile Delta in Egypt, from October to December 1977, causing mortality or abortion in sheep, cattle, domestic buffalo and camels in five governorates, with major economic loss. The epidemic subsided during the cold, winter period, but the disease reappeared in the summer of 1978 and continued until January 1979, spreading to new areas. Retrospective serological studies showed no evidence of RVFV-specific antibodies in domesticated animals or humans in Egypt before the 1977 epidemic. Epidemiological data led to estimates of >20,000 to >200,000 human cases, with *c.* 600 deaths. The unprecedented number of human cases with severe clinical manifestations and the many fatalities

were enigmatic (Laughlin *et al.*, 1979; Meegan, 1979; Arthur *et al.*, 1993). After an interval of 12 years, a new outbreak of RVF occurred in May 1993 in the Aswan Governorate in Southern Egypt, characterized by ocular disease in humans and abortions in sheep, cattle and water buffalo. By August, the outbreak had spread to the Nile Delta (Turell *et al.*, 1996).

During 1982–1985, RVFV was circulating among domesticated animals in southern Mauritania, and at very low rates in five other West African countries (Saluzzo *et al.*, 1987). In October 1987, an outbreak of RVF arose in the Sénégal River basin and grew to a severe epizootic in southern Mauritania and northern Senegal (Digoutte and Peters, 1989; Ksiazek *et al.*, 1989). In Mauritania alone, the outbreak led to many deaths and cases of abortion in livestock, and to an estimated 224 human fatalities (Swanepoel, 1998). Walsh (1988) commented that had there not been additional physicians loaned to the government of Mauritania and working in Rosso hospital, where they were alerted by cases of haemorrhagic fever, the outbreak might have been attributed to yellow fever, which occurs sporadically in the region.

RVFV was first reported outside the African mainland in 1984, following its isolation (named Zinga virus) from pools of mosquitoes collected in Madagascar (Mathiot *et al.*, 1984). Outbreaks of Rift Valley fever occurred in Madagascar in 1990 and 1991 (Morvan *et al.*, 1991, 1992). In 2000, RVF was reported from the Arabian peninsula, where infections in livestock and humans occurred in Yemen and Saudi Arabia. Circumstantial evidence and local experience suggested that there had been a previous outbreak in the Arabian peninsula in 1998 (WHO, 2000b; Jupp *et al.*, 2002).

It is reasonable to surmise that Rift Valley fever virus existed in enzootic transmission cycles in Africa before the appearance of humans and their domesticated animals. The recorded history of Rift Valley fever in mainland Africa and some adjacent territories is incomplete, possibly because some outbreaks of RVF were wrongly ascribed to other haemorrhagic diseases. Certain aspects of the recorded history of RVF are enigmatic; for example, the spasmodic nature of the major outbreaks of disease among domesticated animals, and the differences in human fatality rates in different regions. Possible explanations are offered in the text below.

(d) Genetic variability of RVFV and reassortment

The genome of Rift Valley fever virus, as in other species of *Bunyaviridae*, consists of three segments of RNA which are designated L (large), M (medium) and S (small) (Section 44.1.1.a). The genetic variability of RVFV was investigated by sequencing the coding region of each of the three segments from 20 geographically separate strains. Phylogenetic analyses using the maximum likelihood and maximum parsimony methods revealed the existence of three major lineages corresponding to geographic variants from Central-East Africa, West Africa and East Africa, which were designated Ia, Ib and II, respectively. The segmented nature of the bunyavirus genome permits reassortment, i.e. exchange of an RNA segment when two closely related viruses are present together within the same host cell (Section 44.1.1.c). Comparisons of the phylogenetic trees showed that most groupings of the strains of RVFV matched their geographical sources; however, incongruences indicated that five of the 20 isolates had resulted from reassortment events. Statistical analysis suggested that the incongruences were due to exchanges between strains of different lineages, notably strains from areas of endemicity in West and East Africa. Reassortment under natural conditions implies the presence of two strains, in the same area and at the same time, that are dually infecting the same host cell, whether in a mosquito or vertebrate, with the formation of viable chimeric viruses. In 1993, at least two lineages circulated within Senegal (Sall *et al.*, 1999). Frequent occurrence of such events would have evolutionary significance.

45.6.2 Hosts and vectors of Rift Valley fever virus

(a) Mammals susceptible to infection by RVFV

Serological tests on wild mammals have shown species of five orders to have been naturally infected

with RVFV (Table 45.25). In South Africa, a number of **murine rodents** were found to be susceptible. RVFV-specific antibodies were found in 23% of *Aethomys namaquensis* (n = 312) and 7.7% of *Mastomys natalensis* (n = 65). Fifteen per cent of the *A. namaquensis* sera and 1.5% of the *M. natalensis* sera contained specific neutralizing antibodies of a substantial titre (optical density ≥ 8) (Pretorius *et al.*, 1997). In Senegal, seropositive rodents were found in irrigated farming zones of the Sénégal River valley and in the area Dakar–Niayes to Pout, both within the Sahelian zone, and in the tropical forests of the south. Specific antibodies were present in 4.3% of *Avicanthis niloticus* (n = 140), 13.3% of *Mastomys hildebrandtii* (n = 15), and 2.4% of *Mastomys erythroleucus* (n = 84) (Diop *et al.*, 2000). In Uganda, a single wild-caught *Avicanthis abyssinicus* contained specific neutralizing antibodies (Mims, 1956).

Additional information was obtained through inoculation with RVFV of wild-caught rodents. Individuals of *Avicanthis abyssinicus* developed viraemias lasting 3 or 4 days, with peak titres of $10^{3.7}$–$10^{4.8}$ mouse LD_{50} ml^{-1} blood; they did not succumb to the infection (Weinbren and Mason, 1957). Six *Aethomys namaquensis* developed viraemias but showed no other clinical signs. The viraemias lasted 2 or 3 days, with peak titres of $10^{6.5}$–$10^{8.5}$ $TCID_{50}$ (50% tissue-culture-infective dose) ml^{-1} (Pretorius *et al.*, 1997). Of 27 *Arvicanthis niloticus* and 19 *Mastomys erythroleucus* inoculated with one or other of two strains of RVFV, in only a single *A. niloticus* was a viraemia detected, at $10^{5.7}$ LD_{50} ml^{-1} on day 3 post-inoculation. All laboratory mouse controls became viraemic (n = 80) (Diop *et al.*, 2000). On this evidence, some authors considered that murine rodents are amplifying hosts in enzootic cycles – asymptomatically infected individuals acting

Table 45.25 Mammals susceptible to infection by Rift Valley fever virus.

Rodentia
Muridae; Murinae
Aethomys: *A. namaquensis* (Namaqua rock rat)
Arvicanthis: *A. abyssinicus* (Ethiopian grass rat), *A. niloticus* (African grass rat)
Mastomys: *M. erythroleucus* (Guinea multimammate mouse), *M. hildebrandtii* (Hildebrandt's multimammate mouse; syn. *M. huberti*), *M. natalensis* (Natal multimammate mouse)

Chiroptera
Rhinolophidae; Hipposiderinae
Hipposideros: *H. abae* (Aba roundleaf bat)
Pteropodidae; Pteropodinae
Micropteropus: *M. pusillus* (Peters' dwarf epauletted fruit bat)

Perissodactyla
Rhinocerotidae
Ceratotherium: *C. simum* (white rhinoceros)
Diceros: *D. bicornis* (black rhinoceros)

Artiodactyla
Bovidae; Alcelaphinae
Alcelaphus: *A. buselaphus* (hartebeest)
Connochaetes: *C. taurinus* (blue wildebeest)
Bovidae; Antilopinae
Gazella: *G. granti* (Grant's gazelle)
Bovidae; Bovinae
Bos spp. (domesticated cattle)
Syncerus: *S. caffer* (African buffalo)
Bovidae; Caprinae
Capra: *C. hircus* (domesticated goat)
Ovis: *O. aries* (domesticated sheep)
Bovidae; Reduncinae
Kobus; *K. ellipsiprymnus* (waterbuck)
Suidae; Phacochoerinae
Phacochoerus: *P. africanus* (warthog)

Primates
Cercopithecidae; Cercopithecinae
Cercocebus: *C. torquatus* (red-capped mangabey)
Chlorocebus: *C. aethiops* (vervet monkey)
Erythrocebus: *E. patas* (patas monkey)
Papio: *P. hamadryas* (baboon)
Hominidae
Homo: *H. sapiens* (human)

briefly as cryptic carriers of RVFV. If that is correct, their presumed high population turnover should provide a steady supply of non-immune animals.

In Guinea, RVFV was isolated from the internal organs of **bats** of two species, *Hipposideros abae* and *Micropteropus pusillus* (Boiro *et al.*, 1987).

A serological study of **carnivores** was undertaken in national parks and other locations in Kenya, Tanzania, Zimbabwe, Botswana and South Africa, when most sera found positive by the HI test were checked by plaque reduction neutralization test (PRNT) for an 80% plaque reduction neutralization titre ($PRNT_{80}$). Domestic cats (n = 43), dogs (n = 86), and hyenas (n = 65) were negative to the HI test. A low percentage of cheetahs (n = 64) was positive to the HI test but negative to PRNT. Among black-backed jackals (n = 16), three were positive by HI but the serum samples were insufficient to submit to PRNT. HI-positive sera were obtained from lions (n = 112) from three of four locations, and from two of the locations all HI-positive sera were confirmed by $PRNT_{80}$. In the Ngorongoro Crater area of Tanzania, which is characterized by high rainfall and permanent swamps on the crater floor, 56.3% of lions (n = 16) were positive by PRNT, whereas all lions from the drier Serengeti Plain area (n = 60) were negative. Among lions (n = 31) from the relatively arid Kruger National Park of South Africa, 9.7% were positive to PRNT (House *et al.*, 1996).

In a semi-arid part in Kenya, in an area a few miles from the site of a major epizootic in sheep and cattle in 1968, neutralizing antibody to RVFV was found in small percentages of **game animals** shot during 1968 and 1969: i.e. in 3.2% of blue wildebeest (n = 62); 2.1% of hartebeest (n = 47); and 6.1% of Grant's gazelles (n = 33) (Davies, 1975). Sera collected in the Central African Republic and Democratic Republic of Congo and subjected to HI tests were considered to be specific for RVFV (identified as Zinga virus). Positive results were obtained from 56% of African buffalo (n = 91), 43% of hartebeest (n = 21), 26% of warthogs (n = 43) (cited as *Phacochoerus aethiopicus*, desert warthog) and 40% of elephants (n = 5). No specific antibody was found in wild rodents (n = 123) or birds (n = 113). Digoutte *et al.* (1974a) considered African buffalo to be an amplifying host.

Virological tests showed certain **African monkeys** to be susceptible to infection with RVFV. Inoculation of *Chlorocebus aethiops*, *Cercocebus torquatus* and *Erythrocebus patas* with RVFV induced viraemias, without clinical symptoms, and later the appearance of antibody (Findlay, 1932). Inoculation of baboons (*Papio hamadryas*) induced viraemias that persisted for 3 or 4 days, with little or no malaise, followed some days later by the appearance of antibody (Davies *et al.*, 1972). Serological tests revealed specific antibodies in two of four monkeys screened in the Central African Republic (Digoutte *et al.*, 1974a), but no positive sera were obtained in more extensive studies in East Africa, namely: (i) in monkeys of nine unnamed species (n = 72) in Semliki Forest, Uganda, where RVFV was isolated from mosquitoes (Smithburn *et al.*, 1948); (ii) in *C. aethiops* (n = 1304) trapped in Kenya near areas known to be enzootic for RVF (Davies and Onyango, 1978); and (iii) in baboons (n = 333) at three locations in Kenya during the year after an RVF epizootic (Davies *et al.*, 1972). Humans are susceptible to infection.

The serological studies on wild mammals that are cited above involved relatively small numbers of some species, so the results must be treated cautiously. However, when combined with the experimental virological studies, they suggest that there may be amplifying hosts of RVFV among African rodents and artiodactyls.

Among **domesticated animals**, Rift Valley fever is best known in its epizootic form, which follows the sporadic occurrences of extremely heavy rainfall that leads to the development of aedine vectors in massive numbers. During inter-epizootic periods, small numbers of animals in a herd may become infected with RVFV, and these events are often described as enzootics.

(b) Mosquito hosts and putative vectors

Rift Valley fever virus has been isolated from wild-caught mosquitoes of many species from a range of genera. The following are regarded as the most

probable main and subsidiary vectors in nature (see also Table 45.26).

Subfamily Culicinae
Tribe Aedini
Aedimorphus: *Am. cumminsii*, *Am. ochraceus*, *Am. vexans arabiensis*
Neomelaniconion: *Ne. mcintoshi*
Ochlerotatus: *Oc. caspius*, *Oc. juppi*
Tribe Culicini
Culex (*Culex*): *Cx. antennatus*, *Cx. pipiens*, *Cx. theileri*, *Cx. tritaeniorhynchus*, *Cx. zombaensis*
Culex (*Oculeomyia*): *Cx. poicilipes*.

Systematics: *Aedimorphus*: McIntosh (1975); White (1975); Reinert *et al.* (2009). *Neomelaniconion*: McIntosh (1971); Huang (1985); Zavortink (2004); Reinert *et al.* (2009). *Ochlerotatus*: Reinert *et al.* (2008, 2009). *Culex*: Harbach (2011a,c). Before 1985, *Ne. mcintoshi* was misidentified and cited as *Aedes* (*Neomelaniconion*) *lineatopennis* (now *Ne. lineatopenne*).

In a review, Bouloy (2001) listed 34 species from wild-caught specimens of which RVFV had been isolated; they were species of *Aedimorphus* (cited as *Aedes*), *Neomelaniconion*, *Stegomyia*, *Diceromyia*, *Ochlerotatus*, *Culex*, *Eretmapodites*, *Coquillettidia*, *Mansonia* or *Anopheles*. Species of *Aedimorphus*, *Neomelaniconion* and *Culex* (*Culex*) have been implicated as main or subsidiary vectors on the basis of three types of evidence from the field: (i) isolation of RVFV from wild females; (ii) abundance at the sites of epizootics; and (iii) host preferences in the regions of epizootics (Table 45.26).

Most mosquito species implicated as vectors on evidence from the field were shown to transmit RVFV in the laboratory, but little further information was obtained. A laboratory study was undertaken to estimate the vectorial competence of eight species, of which all but *Cx. pipiens* were tested as P or F_1 adults derived from eggs or adults collected in Africa. Adult females that had fed on viraemic hamsters were examined for dissemination of virus through the body, for the presence of midgut or salivary gland barriers, and for their ability to transmit the virus to uninfected hamsters. Females

Table 45.26 Putative main and subsidiary vectors of Rift Valley fever virus during epidemics of Rift Valley fever among livestock in different regions of Africa and the Arabian peninsula.

Main vectors	*Subsidiary vectors*	*References*
Eastern and Southern Africa		
*Neomelaniconion mcintoshi**	*Aedimorphus cumminsii*	1
Culex theileri	*Neomelaniconion mcintoshi*	2
Culex zombaensis	*Ochlerotatus juppi*	3
	Mansonia uniformis	11
West Africa		
Aedimorphus vexans arabiensis	*Aedimorphus ochraceus*	4, 5, 6
Culex poicilipes		7
Egypt		
Culex pipiens	*Culex antennatus*	8, 9
Ochlerotatus caspius		9
Arabian peninsula		
Aedimorphus vexans arabiensis		10
Culex tritaeniorhynchus		10

*, Originally misidentified and cited as *Aedes lineatopennis* (= *Ne. lineatopenne*).
References: 1, Linthicum *et al.* (1985a,b,c); 2, McIntosh *et al.* (1980); 3, McIntosh *et al.* (1983); 4, Fontenille *et al.* (1995); 5, Fontenille *et al.* (1998); 6. Zeller *et al.* (1997; 7, Diallo *et al.* (2000); 8, Meegan *et al.* (1980); 9, Turell *et al.* (1996); 10, Jupp *et al.* (2002); 11, Sang *et al.* (2010).

of all eight species imbibed RVFV with their blood meals, but midgut-infection and escape barriers and salivary gland barriers reduced, to varying degrees, their capability for transmitting the virus to uninfected hamsters. The estimated transmission rates were: for *Stegomyia* calceata, <2%; *Cx. quinquefasciatus*, <5%; *Ne. mcintoshi*, 5%; *St. aegypti*, 6%; *Neomelaniconion circumluteolum*, 10%; *Cx. antennatus*, 13%; *Cx. pipiens*, 34%; *Ne. palpale*, 39% (Turell *et al.*, 2008a). Among these species, only *Ne. mcintoshi*, *Cx. pipiens* and *Cx. antennatus* are named in Table 45.26 as putative main or subsidiary vectors.

45.6.3 Importance of climate and weather

There is abundant evidence that the spasmodic epizootics and epidemics of Rift Valley fever are associated with periods of exceptionally heavy rainfall. The normal seasonal patterns of rainfall across Africa south of the Sahara result from seasonal movements of the Intertropical Convergence Zone (Subsection 45.6.3.a below), whereas an explanation of the episodes of exceptionally heavy rainfall involves the El Niño–Southern Oscillation (Subsection 45.6.3.b below), which provides medium-term predictive data. A supplementary aid to predictions of periods of heavy rainfall is found in the normalized difference vegetation index (NDVI) (Subsection 45.6.3.c below), which can be calculated from satellite-derived remote sensing data.

(a) Intertropical Convergence Zone

The effect of solar radiation on the temperature of the earth's surface is governed, at each latitude and at each time of year, by the angle of insolation and by day length, adding to their cumulative effects in time. The latitude of maximum solar input is not the equator but those of the two tropics, Capricorn and Cancer. Between 6° N and 6° S of the equator the sun's rays remain almost vertically overhead for only 30 days around each of the spring and autumn equinoxes, allowing little time for the build-up of surface temperature. In contrast, between the latitudes 17.5° and 23.5° N or S of the equator, the sun's rays are almost vertical for 86 days around the time of one of the solstices. Further, day length is longer at these latitudes than at the equator. Owing to a combination of factors, the highest recorded temperatures are at about 23° N and 10–15° S. The latitude at which, at any time during the year, the temperature is maximum is the **thermal equator**. During the year the thermal equator moves cyclically northwards and southwards across the equator, passing through the equatorial and tropical zones and heralding the hot season. The North-east and South-east Trade winds converge at the thermal equator, and rising air currents are produced as a result of the convergence and convection. This band of low pressure and rising air currents is termed the **Intertropical Convergence Zone** (ITCZ).

Over much of Africa south of the Sahara, seasonal rains can be explained by movements of the ITCZ. Along the line of convergence the air necessarily rises; and while rising its volume and pressure change and the air cools without loss of heat to the surrounding air (adiabatic cooling). This cooling often leads to condensation of moisture, cloud formation and rain, producing a 'convective rainfall' belt. During the year, driven by the movement of the thermal equator, the ITCV moves cyclically northwards and southwards across the equator, bringing cloudy weather and rain. It crosses the equatorial zone twice each year, but reaches the northern and southern extremities of its range only once (Figure 45.27). In January, the ITCZ dips U-shaped into southern Africa, as far as 20° S over Botswana and Zimbabwe, and by July it has moved to 15° N, at the southern fringe of the Sahara (Barry and Chorley, 1998).

In Kenya there are two rainy seasons during the year, coinciding with the position of the ITCV. In Nairobi, at 1° 20' S, a short rainy season usually lasts from October to December while the ITCV is moving southwards, and a long rainy season, with over twice the rainfall, lasts from March to June while it is moving northwards (Linthicum *et al.*, 1990).

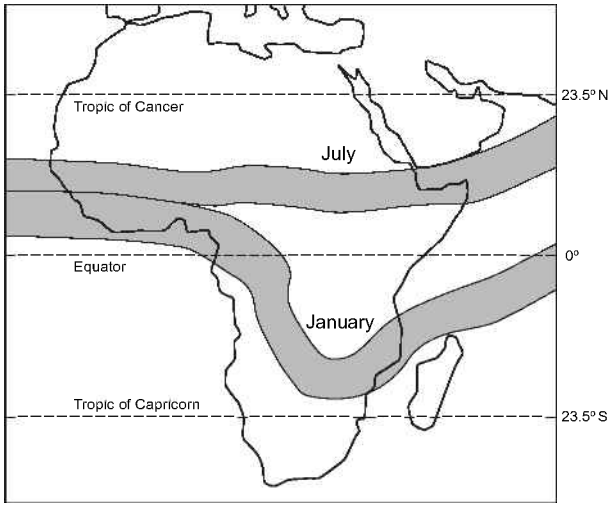


Figure 45.27 Seasonal movements of the Intertropical Convergence Zone across Africa. In January, the tropical rainy zone is well south of the equator, but as the thermal equator moves northwards so do the rains, and by July they lie across Mali, southern Chad and Ethiopia. (After File, 1991.)

(b) El Niño–Southern Oscillation

Fundamental to this topic are the **Walker circulations**, east–west tropical airflows driven by major east–west gradients of atmospheric pressure that result from differences between air rising over heated continents and the warmer parts of the ocean, and air subsiding over cooler parts of the oceans and over certain continental areas. These circulations were first observed over the eastern Pacific Ocean and Indonesia. Normally, a low atmospheric pressure develops in the region of northern Australia, and a high pressure system over the coast of Peru. As a result, the easterly trade winds move strongly from east to west over the equatorial Pacific Ocean, skimming water off the surface of the Pacific where warm surface water accumulates and shifting it towards the western Pacific Ocean. As a result, a current of cold Antarctic water called the Peru (or Humboldt) Current flows northwards up the coastlines of Chile and Peru, where the low sea-surface temperatures (SSTs) produce a shallow inversion, thereby strengthening the trade winds (Figure 45.28, A).

That is the positive phase of the tropical Pacific Ocean Walker Circulation. During the occasional negative phase, which occurs at intervals of up to 10 years, the pattern of atmospheric pressure over the Pacific is reversed, and the area of low pressure and convection is displaced eastwards towards the 180° meridian in the middle of the Pacific Ocean (Figure 45.28, B). The occurrence of changing positive and negative phases of the Pacific Walker Circulation is termed the **Southern Oscillation**.

The Southern Oscillation reflects a see-sawing standing wave of atmospheric mass and pressure involving exchanges of air between subtropical high- and low-pressure cells over the equatorial region of the Pacific Ocean. The strength of the Southern Oscillation is indicated by the Southern Oscillation Index (SOI), a comparison of atmospheric pressure at Tahiti (150° W, 18° S) with that at Darwin in Australia (130° E, 12° S) as expressed by a standardized deviation from the norm. Thus,

$$SOI = 10.0 \times \frac{[SLPdiff - avSLPdiff]}{StdDev(SLDdiff)} \qquad (45.4)$$

where *SLP* = sea level pressure; *SLPdiff* = (mean Tahiti *SLP* for the month) – (mean Darwin *SLP* for the month); *avSLPdiff* = long-term mean of *SLPdiff*

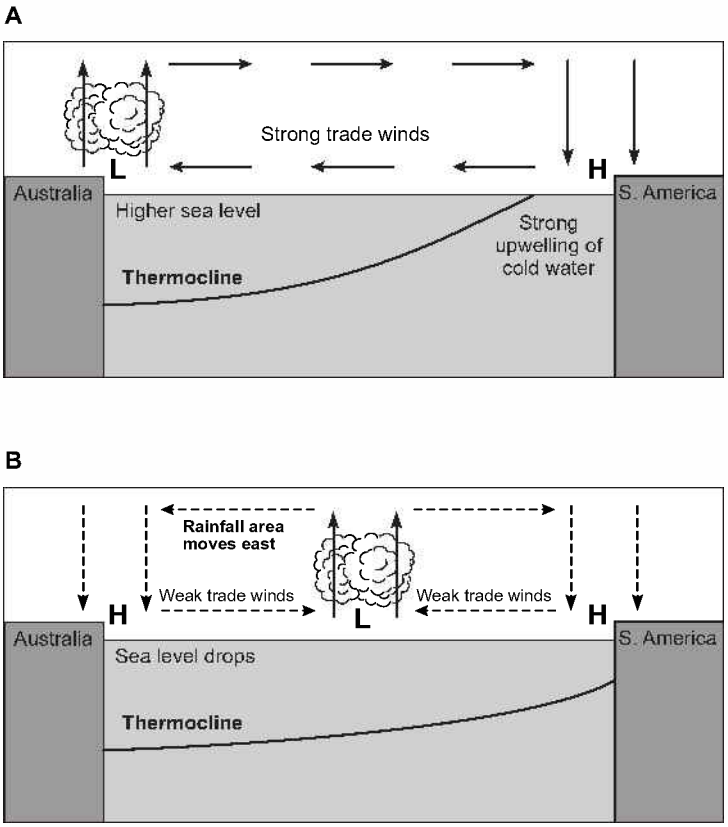


Figure 45.28 El Niño Southern Oscillation. Cross-section of the Pacific Ocean at the latitude of the equator, illustrating two different patterns of atmospheric circulation and surface-water temperature. (From PhysicalGeography.net. http//:www.physicalgeography.net/.) **A** During a normal (non-El Niño) year. The atmospheric pressure is low in the region of northern Australia and Indonesia, but high over the coast of Peru. As a result, the trade winds over the Pacific Ocean move strongly from east to west, driving warm sea-surface waters westwards. This allows cold bottom water to well up to the surface along the coastlines of Peru and Ecuador. **B** During an El Niño event. Atmospheric pressure falls over large areas of the central Pacific Ocean. The normal low atmospheric pressure over the western Pacific is replaced by a weak high, a reversal termed the Southern Oscillation. The change in pressure pattern causes the force of the trade winds to decline, allowing an 'equatorial counter current', which runs west to east, to accumulate warm water along the coastlines of Peru and Ecuador. This accumulation of warm water causes the thermocline* to drop in the eastern Pacific Ocean, which reduces the upwelling of deep and cold ocean water. *, Thermocline: temperature gradient in a body of water separating layers at different temperatures. L, low atmospheric pressure. H, high atmospheric pressure.

for the month in question; *StdDev*(*SLPdiff*) = standard deviation of *SLPdiff* for the month in question.

The ocean is a massive source of heat energy. A pool of warm surface water, 100 m or more deep, can build up in the western equatorial Pacific owing to intense insolation, low heat loss from evaporation in a region of light winds, and the piling up of warm surface water driven westwards by the easterly trade winds. During most years, and usually starting in December, there is a southwards flow from that body of warm water. Near the coast of South America it meets and is blocked by the northwards flowing Peru Current (Figure 45.28, A), and its pool of heat energy is dissipated by the changing ocean currents and by release into the

atmosphere. In those years the limited southward flow of warm sea water is named **La Niña**.

During the negative phase of the Pacific Walker Circulation, which occurs at intervals of 2 to 10 years, and on average of 4 years, when the pattern of atmospheric pressure over the equatorial Pacific is reversed, the surface water in the eastern equatorial Pacific becomes much warmer, and the southward flow from that body of water, now named **El Niño**, causes the coastal upwelling of the cold water of the Peru Current to cease entirely (Figure 45.28, B). The term **El Niño–Southern Oscillation** (ENSO) is used for that event and the climatological events that ensue. Under ENSO conditions there is a very much greater release of heat energy into the atmosphere, leading to perturbations of climate over vast distances, including accentuated precipitation over Amazonia, Africa, India and Indonesia (Barry and Chorley, 1998).

More generally, large-scale convection over the warm tropical oceans provides an important portion of the driving energy for the circulation of the atmosphere. Sea-surface temperatures in excess of 27.5°C seem to be the threshold for organized convective activity, and over broad regions of the Pacific and Indian Oceans such SSTs cause large-scale convection (Graham and Barnett, 1987; Barry and Chorley, 1998). Climatological cycles and patterns such as ENSO modulate the background environment, and tropical systems are then moved by steering winds in the troposphere. There is strong evidence that Indian Ocean surface temperatures influence the climate of southern Asia and Africa. For example, warming influences rainfall over the Indian Ocean itself and over eastern Africa. When Indian Ocean SSTs are elevated but those of the Pacific Ocean are not, rainfall in East Africa is enhanced to a degree, producing localized rainfall that may lead to focal Rift Valley fever activity but no more. High Indian Ocean SSTs may occur during periods of elevated SSTs in the eastern Pacific associated with El Niño events, when the combined influences can result in widespread, heavy rainfall over eastern Africa, leading to epizootics/epidemics of RVF. Sea-surface temperatures are routinely measured with Advanced Very High Resolution Radiometer (AVHRR) instrumentation on satellites (Table 45.27).

(c) Normalized difference vegetation index

In semi-arid areas, rainfall and abundance of green vegetation are necessary for many insect species to enter an active state, and in such areas development of the aquatic stages of mosquitoes is closely linked with rainfall events, for which vegetation growth is a good indicator. The normalized difference vegetation index (NDVI) is a numerical indicator used to analyse data produced by remote sensing instruments on satellites, to determine whether or not a target area contains abundant chlorophyll, an indicator of green vegetation (Table 45.27). Rainfall patterns and changes in ground moisture can be determined from the temporal and spatial dynamics of green-leaf vegetation, for example by measuring soil and canopy reflectances and transmission.

The wavelengths of solar radiation absorbed by chlorophylls a and b are designated as 'photosynthetically active radiation' or PAR, and in the chlorophyll absorption spectrum are apparent as two major absorption peaks, at 430–475 nm within the blue band, and at 650–670 nm within the red band. Incident light of those wavelengths is absorbed by chlorophyll, the photons providing energy for the primary photochemical process. Radiation of all other wavelengths, including the infrared (740–1500 m), is reflected. For that reason, green plants appear relatively dark in the PAR spectral region but relatively bright in the near infrared. The NDVI is defined as follows:

$$NDVI = \frac{\rho_{nir} - \rho_{red}}{\rho_{nir} + \rho_{red}} \qquad (45.5)$$

where ρ_{nir} and ρ_{red} represent reflectance at near infrared and red wavelengths (Matsushita *et al.*, 2007). When recorded from different sites, differences between spectral reflectances measured in the red and the near-infrared regions provide ratios of the reflected over the incoming radiation in each spectral band, with values between 0.0 and 1.0. This enables calculation of NDVIs, which vary

Table 45.27 Instrumentation used in remote sensing programmes developed for investigations of Rift Valley fever epidemiology, with descriptions of the satellites that carry them. (From data in Pope *et al.*, 1992; Matsushita *et al.*, 2007.)

High spatial resolution sensors

Carried on satellites (space-borne systems) or on aircraft (airborne systems).

AVHRR. Advanced Very High Resolution Radiometer; space-borne. Used to measure sea-surface temperatures, from which future rainfall patterns can be predicted.

MODIS. Moderate Resolution Imaging Spectroradiometer. Carried on Terra (EOS AM) and Aqua (EOS PM) satellites. Captures data in 36 wavelength bands between 0.4 and 14.4μm, obtaining data from land, oceans and lower atmosphere, including wavelengths indicative of photosynthetic activity.

SAR. Synthetic Aperture Radar. Airborne. The micro-scale wavelengths generated by SAR sensors can penetrate clouds and emergent radiation. High resolution L and C band multipolarization SAR imagery was effective in detecting flooded dambos, but LHH was the best channel for discrimination between flooded and non-flooded sites in sedge and short grass environments.

TM. Thematic Mapper. Optical sensors used in Landsat TM (see below), with six visible and infrared bands of high resolution (~30 m). Used to identify potential mosquito larval habitats, but use is hindered by cloud cover and by emergent vegetation that obscures water surfaces.

Satellites used to carry sensors

Landsat. Satellites launched by NASA (US *National Aeronautics and Space Administration*) to acquire high resolution (500–800 m) remotely sensed multispectral images of the earth's land surfaces and oceans. Equipped with Thematic Mapper (TM) that scans in seven spectral bands from visible to thermal infrared, and with Multispectral Scanner (MS) that acquires images in four spectral bands from visible to reflected infrared. Primarily used to monitor atmospheric and oceanic conditions, including sea-surface temperatures, and ecological conditions. Have been equipped with sensors that respond to reflected sunlight and infrared radiation.

NOAA. US satellites used to monitor the earth and oceans, providing information on sea-surface temperatures. Also used to observe vegetation dynamics.

SPOT. French satellites (Satellite Pour l'Observation de la Terre). Normally used to explore the earth's resources, to detect and forecast climatological and oceanographic phenomena, and to monitor natural phenomena.

TERRA (EOS AM) and AQUA (EOS PM). US satellites that orbit the earth. Terra is timed to pass from N to S across the equator in the morning, and Aqua to pass S to N across the equator during the afternoon, the instruments scanning the earth's entire surface every 1–2 days.

between −1.0 and +1.0. The NDVI is a reliable indicator of rainfall, and a correlation was found between high NDVI values and flooding of mosquito larval habitats. Relatively small water bodies in which mosquitoes develop can be identified with satellite-borne instruments of high spatial resolution (Tucker *et al.*, 1985b; Linthicum *et al.*, 1987, 1990; Pope *et al.*, 1992). In East Africa, analyses of surveys over zones of forest, woodland, bush and grassland revealed an overall log-linear relationship between NDVI and rainfall. The correlation between annually integrated NDVI and the log of annual rainfall at 65 stations was 0.89 (Davenport and Nicholson, 1993).

(d) Climate and epizootics/epidemics of RVFV

In eastern and southern Africa, in years of near-average rainfall, the rain produces relatively small water bodies from which mosquito populations of moderate size emerge that are thought to maintain enzootic cycles of Rift Valley fever in wild mammals. In contrast, in years of exceptional rainfall, larger water-filled bodies appear, providing aquatic habitats from which mosquitoes emerge in numbers sufficiently massive to initiate epizootic cycles of RVFV in domesticated animals. All major outbreaks of RVF recorded in Kenya from 1950 to 1998 followed periods of abnormally heavy rainfall,

which were associated with the development of an exceptionally pronounced Intertropical Convergence Zone. Retrospective analyses, especially that of the 1997–1998 ENSO, showed that satellite-measured indices of climate could be used to predict outbreaks of RVF up to 5 months in advance. The development of warm ENSO conditions, indicated by sea-surface temperatures greater than normal by >1°C in the eastern-central Pacific Ocean and by >0.5°C in the western-equatorial Indian Ocean during the period September to November 2006, enhanced precipitation over regions of the Pacific Ocean and over the western Indian Ocean extending into the Horn of Africa. Coupled with NDVI results indicating deviations from the usual, these data had useful predictive value (Linthicum *et al.*, 1999).

Each of seven moderate or large RFV outbreaks that occurred in the Horn of Africa (Eritrea, Djibouti, Somalia and Ethiopia) from 1950 to 2009 was associated with widespread and above normal rainfall due to the El Niño–Southern Oscillation. ENSO-related climate anomalies were analysed, using a combination of data for elevated sea-surface temperatures, subsequent elevated rainfall and NDVI. From those data, an RVF risk-mapping model predicted areas where outbreaks of RVF in domesticated animals and humans were expected in the Horn of Africa from December 2006 to May 2007. The predictions were later confirmed by entomological and epidemiological field investigations of virus activity in the areas identified as at risk (Anyamba *et al.*, 2009).

In West Africa, most research on transmission of RVFV has been undertaken within the Sahel region of Senegal. More broadly, the Sahel is a semi-arid region that extends from Senegal eastwards to Sudan, forming a narrow transitional band between the humid savannahs to the south and the arid Sahara to the north. In Senegal, a gradient of rainfall declines from the south to the north of the Sahel at approximately 1 mm km^{-1}, while annual rainfalls vary from 600 mm in the south to 100 mm in the north. The length of the mono-modal rainy season varies from 3.5 months in the south (mid-June through to September) to 1.5 months in the north. At that time, the ITCZ is at its most northern latitude, and no further rain falls until the following year. Monitoring vegetation dynamics by the AVHRR sensor on a US NOAA satellite showed a correlation between seasonal variations in the density and extent of green-leaf vegetation and the patterns of rainfall associated with movement of the ITCV. Most falls of rain occurred as squall lines that moved rapidly westwards. Observed by radar, each squall line was composed of two different entities: a narrow line of thunderstorm cells which could extend for hundreds of kilometres, generating rainfall of up to 100 mm h^{-1}, but of short duration, followed by a wide area of light stratiform rain of <10 mm h^{-1}, which was of longer duration (Tucker *et* al., 1985a,b; Buarque and Sauvageot, 1997; Barry and Chorley, 1998). Unfortunately, there have been no productive investigations into the relationship between climate and RVF prevalence. The development of a stochastic, non-linear method of mapping mosquito vectors in the Barkedji area in northern Senegal and RVF prevalence in a host population in relation to variations of rainfall produced inconclusive findings (Bicout and Sabatier, 2004).

45.6.4 Transmission of RVFV in eastern and southern Africa

(a) Aquatic habitats of putative vectors

Of the putative vectors of RVFV, the species of Aedini make use of temporary, rain-filled pools and of some more permanent pools, while the species of Culicini are associated with semi-permanent and permanent water bodies – ditches, pools in swamps or river beds, and borrow pits. Enzootic transmission occurs during periods of normal rainfall when the vectors develop in water bodies of relatively small size, whereas epizootics of Rift Valley fever follow periods of exceptionally heavy rain and the filling of very large aquatic habitats.

Certain species of *Neomelaniconion* and *Aedimorphus* lay desiccation-resistant eggs in shallow but extensive depressions in the ground. In eastern

Africa these are **dambos** – shallow, streamless depressions, which range from a few metres to several kilometres in length, situated at the headwaters of drainage systems. Dambos often are found in areas of grassland, but they occur also in primary forest, where the surrounding trees and shrubs stop at the dambo margin. They remain dry for long periods, even years, and become flooded when persistent, heavy rainfall raises the level of the water table, but stay flooded for a period of only weeks (Linthicum *et al.*, 1983). In Central Province, Kenya, the only ecological change that increased the surface area of standing water, and produced conditions necessary for production of large numbers of mosquitoes, was flooding of the dambos (Linthicum *et al.*, 1984a). That substantial rainfall is needed to fill these depressions became apparent during the study of a particular dambo; over a period of 3 days there was an accumulation of 109 mm rainfall, but even so the dambo did not flood (Logan *et al.*, 1991). In South Africa, similar large seasonally flooded shallow depressions in grassland, with no outlets, provide important larval habitats for aedine mosquitoes, but there they are called **pans** (Gargan *et al.*, 1988).

Soon after the flooding of dambos or pans, dormant eggs of the aedine species hatch and give rise to massive numbers of adult mosquitoes. Later, other mosquito species make use of the dambos as larval habitats. Daily sampling of an artificially flooded dambo in Kenya showed the numbers of 1st and 2nd instar *Am. cumminsii* larvae peaking at 2 days after flooding, and those of *Ne. mcintoshi* peaking at 6 days. *Culex* larvae peaked at 9–13 days post-flooding (Linthicum *et al.*, 1984a). In a naturally flooded dambo, the total larval count of *Am. cumminsii* peaked at 3–10 days, that of *Ne. mcintoshi* at 10–14 days. *Culex quasiguiarti* larvae first appeared 15 days after flooding, but their numbers did not build up until some 15 days later. *Culex theileri* larvae first appeared 33 days after flooding. All of the aedine species disappeared after a single generation, but the *Culex* species appeared to produce multiple generations (Linthicum *et al.*, 1983).

Artificial flooding of an area of 1800 m^2 within a dambo induced a hatch of aedine eggs, and produced an estimated 1.3×10^6 female and 1.2×10^6 male pupae of *Ne. mcintoshi*. From an estimated 35% survival rate from pupae to adults, it was estimated that 455,000 adult female and 420,000 adult male *Ne. mcintoshi* emerged (Linthicum *et al.*, 1985a). In areas of Kenya where RVF occurs, herdsmen graze their cattle in and around dambo formations (Linthicum *et al.*, 1984b). The period of survival of dormant *Neomelaniconion* eggs in the field is not known. When a mixture of field-collected eggs of *Neomelaniconion* (90% *Ne. mcintoshi*, 11% *Ne. circumluteolum*) were kept at 24°C for 9 months, 5% hatched, of which 71% developed to adults. Kept at 26°C for 9 months, 16% of eggs hatched, 83% of which developed to adults (Logan and Linthicum, 1992).

(b) Adult biology of putative vectors

Little is known of the adult biology of most putative enzootic vectors of RVFV. If both murid rodents and bovids can be amplifying hosts, mosquito species with different host preferences must serve as vectors, and probably in separate transmission cycles.

An investigation into host preferences was conducted within an outbreak area near Nairobi where cattle were the predominant mammals but other domesticated and wild animals were also present. A period of heavy rainfall, from October to December 1982, led to the development and emergence of a number of aedine species, but did not induce an epizootic. Among engorged females of six aedine species caught in light traps, the proportions of identifiable blood meals that had been obtained from farmed cattle exceeded 94% for five of the species, and exceeded 84% for the sixth. The other identified blood meals came from humans, canines, rabbits, giraffes, equines and unidentified bovids (Linthicum *et al.*, 1985c). This result might be taken to indicate a very high preference of all six species for cattle as hosts, but use of the forage-ratio technique would have given different and more realistic estimates. Determination of the forage ratio of a mosquito species requires knowledge not only of the numbers of blood meals taken on different hosts, but also of

the number of individuals of each host species within the area occupied by the mosquito population, and of the total number of potential hosts within the area (Volume 2, Section 39.2.2).

Neomelaniconion mcintoshi, which was the most abundant mosquito in that investigation, was also the most abundant at a dambo in Thika District, Kenya, where catches on bovine and human bait were recorded. When one calf was exposed for 48 h, 878 mosquitoes of seven species were caught (18 h^{-1}). When two men were exposed for 96 h, 1446 mosquitoes of nine species were caught (15 h^{-1}). *Neomelaniconion mcintoshi* constituted 95.1% of the catch on the calves, and 85.4% of the catch on humans, which suggests equivalence between one calf and two men. *Aedimorphus cumminsii* formed 4.1% of the catch on humans, but only 0.3% of the catch on the calf (Linthicum *et al.*, 1984b).

The life expectancy of *Ne. mcintoshi* is sufficient for the females to be effective vectors. Totals of 62,463 adult female and 25,048 adult male *Ne. mcintoshi* were collected during a period of 6–7 weeks after their emergence from a flooded dambo. Regression of numbers captured against time since emergence suggested that the daily survival rate was independent of age. For males, the daily survival rate was determined as 0.83 (95% c.i. 0.82–0.84); for females the rate was 0.85 (95% c.i. 0.84–0.86). Females were captured up to 44 days post-emergence, during which period the overall mean distance travelled was 0.15 km, generally in the direction of the prevailing wind (Linthicum *et al.*, 1985a).

(c) *Vertical transmission*

Both circumstantial and more direct evidence suggests that vertical transmission allows RVFV to survive through long dry periods within the desiccation-resistant eggs of aedine mosquitoes. (i) In Kenya, RVFV was isolated from *Ne. mcintoshi* that had been collected as larvae from both naturally and artificially flooded dambos and reared to adults. The MIRs/1000 were: ♂♂, 0.625–1.37 (n = 2331); ♀♀, 1.16–3.58 (n = 4008) (Linthicum *et al.*, 1985b). (ii) After exceptionally heavy rainfall, when large populations of mosquitoes had developed, epizootics of Rift Valley fever occurred simultaneously in locations separated by several hundred kilometres. These large populations were of aedine mosquitoes, able to persist in arid areas as dormant eggs.

Vertical transmission of RVFV from generation to generation through its aedine hosts may contribute to the maintenance of enzootic transmission cycles. It may also enable RVFV to survive through inter-epizootic periods in habitats which, when flooded, will permit the rapid production of massive mosquito populations. Taking the simulation model of transmission of Keystone virus (Section 41.4.2) as a guide, the initiation of epizootics among young farmed ruminants by the bites of vertically infected *Ne. mcintoshi* emerging from flooded dambos appears feasible. However, a number of key factors remain to be established, e.g.: the prevalence rate of vertical infection among newly emerged mosquitoes; the proportion of blood meals taken on susceptible and potentially viraemic hosts; the number of vectors per susceptible host at the time of initial blood feeding; and the probability of a vector surviving through a gonotrophic cycle. Quantitative data are needed to demonstrate: (i) an effective incidence rate of infections in the host population after the first mosquito blood meals (Eqn 41.4); and (ii) a sufficient incidence rate of infections in the mosquito population at the time of the second blood meal (Eqn 41.5).

(d) *Enzootic and inter-epizootic transmission in eastern and southern Africa*

Wild amplifying hosts have been sought among the mammalian fauna of forests and more open habitats, and the possibility of enzootic transmission among flocks and herds of farmed animals has also been examined. In wild animals, infections with RVFV are asymptomatic so evidence of infection has been almost entirely restricted to serological data, which do not differ between amplifying and dead-end hosts. The evidence is,

therefore, difficult to interpret. Serum samples have been taken from a wide range of species. Most early searches for RVFV-specific antisera among wild birds, primates and rodents produced negative results, possibly because most species were represented by small samples (Smithburn *et al.*, 1948; Swanepoel *et al.*, 1978; Davies and Addy, 1979). In contrast, more recent searches have been more productive.

RVFV-specific antibodies have been identified in murine rodents, notably in species of *Avicanthis*, *Aethomys* and *Mastomys*. In South Africa, where serum samples were taken from small terrestrial mammals captured in known enzootic areas before and after the 1988 floods, the highest antibody activity was found in the Namaqua rock rat, *Aethomys namaquensis*. Among samples taken before the 1988 floods (n = 141), 6.4% were seropositive, whereas, among sera taken after the 1988 floods (n = 171), 22% were positive (p = 0.001). The rocky outcrops that are the natural habitat of *A. namaquensis* are not normally situated near water sources, where the highest concentrations of mosquitoes are found. Pretorius *et al.* (1997) surmised that *A. namaquensis* serves as a cryptic carrier of RVFV during inter-epizootic periods, and suggested that the higher prevalence rate that had been found among females after the floods reflected amplification.

Mosquito species from which RVFV has been isolated during inter-epizootic periods may or may not be main or subsidiary enzootic vectors. In Kenya, during 3 years within an inter-epizootic period (October 1981 to October 1984), 108,026 mosquitoes captured in three ecologically different zones were pooled and tested. Isolations of RVFV were made from species of *Aedimorphus*, *Neomelaniconion*, *Culex* and *Anopheles* (Table 45.28) (Linthicum *et al.*, 1985b). One of these, *Culex rubinotus*, was said to feed mainly on wild rodents (McIntosh *et al.*, 1980). *Aedimorphus cumminsii* and *Ne. mcintoshi* are possible enzootic vectors and putative epizootic vectors. During the 1940s, in an investigation at Mongiro in Semliki Forest, Uganda (cf. Section 45.3.3.a), RVFV was isolated from three pools of *Eretmapodites* spp., one pool of *Stegomyia* spp., and two pools of the *Catageiomyia tarsalis* species group (Smithburn *et al.*, 1948). Davies (1975) considered that Rift Valley fever was enzootic in Semliki Forest.

Table 45.28 Species of mosquitoes from which Rift Valley fever virus was isolated during inter-epizootic periods in East and West Africa. In Kenya, mosquitoes were captured in light traps, in sweeping nets and at human bait (Linthicum *et al.*, 1985b). In Senegal, mosquitoes were captured in CO_2–light traps and at human and animal bait placed near ground pools and cattle droves (Zeller *et al.*, 1997).

Species	*Total tested* (n)	*Pools*		
		Tested	*Positive*	*MIR*
Kenya, 1981–1984				
Aedimorphus cumminsii	2,997	116	1	0.33
Neomelaniconion mcintoshi	59,644	563	9	0.15
Culex (Cux.) antennatus	4,988	226	3	0.60
Culex (Cux.) vansomereni	2,476	103	1	0.40
Culex (Eum.) rubinotus	1	1	1	
Anopheles (Cellia) chrystyi	827	78	2	2.42
Anopheles (Cellia) pharoensis	1	1	1	
Senegal, 1991–1993				
Aediomorphus ochraceus	2,185	126	3	1.37
Culex (Ocu.) poicilipes	14,937	299	26	1.74

MIR, minimum infection rate.

The occurrence of sporadic infections with RVFV in young farmed ruminants during seasons with normal or low rainfall has been taken by some authors to indicate involvement in enzootic transmission during inter-epizootic periods, as observed, for example, in South Africa on the temperate inland plateau west of the Drakensberg escarpment, and in the subtropical coastal lowlands of Natal (McIntosh *et al.*, 1980). In Kenya, seroconversions were found in low percentages of cattle in forest-edge situations. Davies (1975) ascribed these to transmission from a cryptic enzootic carrier, but considered that there was no clear evidence of farmed ruminants serving as amplifying hosts during an inter-epizootic period (Davies, 1975).

To identify environmental risk factors for humans during inter-epizootic periods, an investigation was carried out in Kenya in 2006, some 8 years after the El Niño–related flooding and RVF epizootic there in 1997–1998. Serological surveys were conducted in two areas of Ijara District (c. 1° 40' S, 40° 10' E) in a semiarid region of the Northeastern Province. Screening for serum anti-RVFV IgG revealed an overall seropositivity rate of 13% (n = 248). However, following infection with RVFV, anti-RVFV IgG antibodies may persist in the serum indefinitely (Section 44.3.4.d), and do not indicate the date of infection. In this investigation, evidence for infection with RVFV during the inter-epizootic period was provided by the 47.6% seropositivity rate in children of up to 15 years of age, the youngest of whom to be found seropositive was aged 4 years. For each year of life, the human seropositivity rate increased by 5%. To what extent the source of the virus was wild amplifying hosts was uncertain (LaBeaud *et al.*, 2008).

(e) Epizootics/epidemics in livestock and human populations in eastern and southern Africa

Among **domesticated animals**, Rift Valley fever is a disease of sheep, goats, cattle, buffalo, camels, horses and donkeys. Most domesticated ruminants are amplifying hosts. Usually, viraemia is demonstrable at the onset of fever, and can persist for up to a week. Maximum recorded virus titres (as mouse LD_{50} ml^{-1}) were $10^{10.1}$ in lambs, $10^{8.2}$ in kids and $10^{7.5}$ in calves. Somewhat lower maximum titres were recorded from adult animals (Swanepoel, 1998; Boden, 2001). In duplicated experiments in which lambs were inoculated with 10^5 PFU of RVFV, the peak viraemias in 3-day-old lambs were $10^{6.2}$ and $10^{7.0}$, and in 6–8-week-old lambs were $10^{5.0}$ and $10^{5.3}$ PFU ml^{-1}. Viraemias were first detected after about 24 h, were highest between 28 and 52 h, and were barely detectable by 72 h post-inoculation. In 13 out of 14 experiments, the median viral titre recovered from engorged *Cx. pipiens* was $>10^{2.0}$ PFU (Turell *et al.*, 1984b).

Epizootics and epidemics of Rift Valley fever in livestock and human populations have occurred during the sporadic periods of exceptional rainfall associated with an El Niño–Southern Oscillation (Section 45.6.3.d). For example, in 1974 and 1975, unusually heavy rains fell over most of South Africa, filling the pans in the central regions, and an epizootic of RVF led to the deaths of thousands of lambs and hundreds of sheep and cattle, while many human cases occurred (Van Velden *et al.*, 1977). Also, an epidemic that started in Kenya in December 1997 and that spread to Somalia and northern Tanzania, causing an estimated 89,000 human infections, was associated with the worst flooding in the region since 1961 (Sall *et al.*, 1998; WHO, 1998). The outbreaks arose in places where very large populations of a main vector had appeared. In Kenya, the two putative vectors of RVFV, *Ne. mcintoshi* and *Am. cumminsii*, could be captured in small numbers during normal rainy seasons when only temporary ground pools were filled, but they appeared in massive numbers when dambos flooded (Subsection 45.6.4.a above). Five species of *Culex*, including the putative vectors *Cx. theileri* and *Cx. zombaensis*, could be captured throughout the year in small numbers, but in much higher numbers during the rainy seasons, and particularly when the dambos filled with water (Linthicum *et al.*, 1984a).

More recently, starting in December 2006 and continuing until June 2007, a very extensive outbreak of RVF across six of the eight provinces of

Kenya affected thousands of animals (cattle, sheep, goats and camels). Approximately 700 human cases of RFV were reported (Munyua *et al.*, 2010). In January 2007, an outbreak of RVF was detected among humans in north-central and southern Tanzania. Some 511 suspect human cases were recorded clustering around areas of livestock infection, with 186 confirmed cases leading to a 28.2% death rate, and another 123 probable cases. The inland plateau that was heavily affected by the outbreak is usually hot and dry, but during the period December 2006 to June 2007 excessive rainfall led to flooding in most parts of the plateau, including the 'breeding habitats' of aedine mosquitoes (Mohamed *et al.*, 2010).

Outbreaks of RVF in Africa have been associated with cyclical patterns of the El Niño–Southern Oscillation phenomenon (Section 45.6.3.b). Satellite-derived measurements of global and regional elevated sea-surface temperatures, elevated rainfall and NDVI allowed successful predictions, with lead times of 2–4 months, of where outbreaks of RVF should occur in eastern and southern Africa from September 2006 to March 2008 (Anyamba *et al.*, 2010).

Two means by which epizootics might start in populations of domesticated animals have been postulated. First, vectors involved in a forest enzootic cycle infect farmed animals on adjacent grasslands. Meegan and Bailey (1989) recorded epizootics of RVF among domesticated ruminants grazing in bushed and wooded grasslands. Seropositivity rates show that in such situations the prevalence of infection is usually low and epidemiologically unimportant, but the emergence after heavy rain of large numbers of mosquitoes of species that colonize temporary aquatic habitats opportunistically, and that have vectorial capabilities, makes an epizootic possible (Davies, 1975). Infections with RVFV can spread rapidly through flocks of sheep, and at rates suggestive of mechanical transmission by vectors (Jupp *et al.*, 1984). Experimentally, RVFV has been mechanically transmitted to lambs on the mouthparts of mosquitoes (Section 44.4.2). Transmission to humans by the bite of infective mosquitoes is possible but has not been demonstrated.

A second way in which epizootics might start is the filling of dambos or pans, triggering hatching of aedine eggs, some of which are vertically infected with RVFV (Subsection 45.6.4.c above). Vertically infected adult females might transmit RVFV to small numbers of livestock, and the later emergence in large numbers of other vector species might allow the establishment of an epizootic/epidemic (Figure 45.29).

Cases of RVF among humans mostly result from close contact with diseased animals. In the 1997–1998 heavy flooding in East Africa, the epicentre of the Kenyan epidemic was the Garissa District, near the border with Somalia, where there were an estimated 27,500 new human infections. A serological survey of 171 susceptible individuals in Garissa District showed 31 (18%) to be positive for RVFV-specific IgM, indicating recent infections, while 140 (82%) were seronegative. A variety of forms of contact with animals was recorded among those 171 individuals, of which herding, milking, slaughtering and sheltering animals in the home were statistically associated with recent RVFV infection (Table 45.29). Relative risk of infection was greatest among activities involving contact with sheep (Woods *et al.*, 2002). In the 2006 to 2007 outbreak of RVF in Kenya, contact with sick animals, and with animal products including blood, meat and milk, was identified as a major risk factor (Mohamed *et al.*, 2010).

45.6.5 Transmission of RVFV in West Africa

Most knowledge of RVFV transmission in West Africa comes from investigations in Senegal, and especially from research undertaken in two areas, both within the Sahelian bioclimatic region. One is the Sénégal River basin, which is situated in the north of Senegal and south of Mauritania, the Sénégal River forming a boundary between those two countries. The other lies around Barkedji, which is situated in northern Senegal and is in a region of seasonal pasture (Figure 45.30).

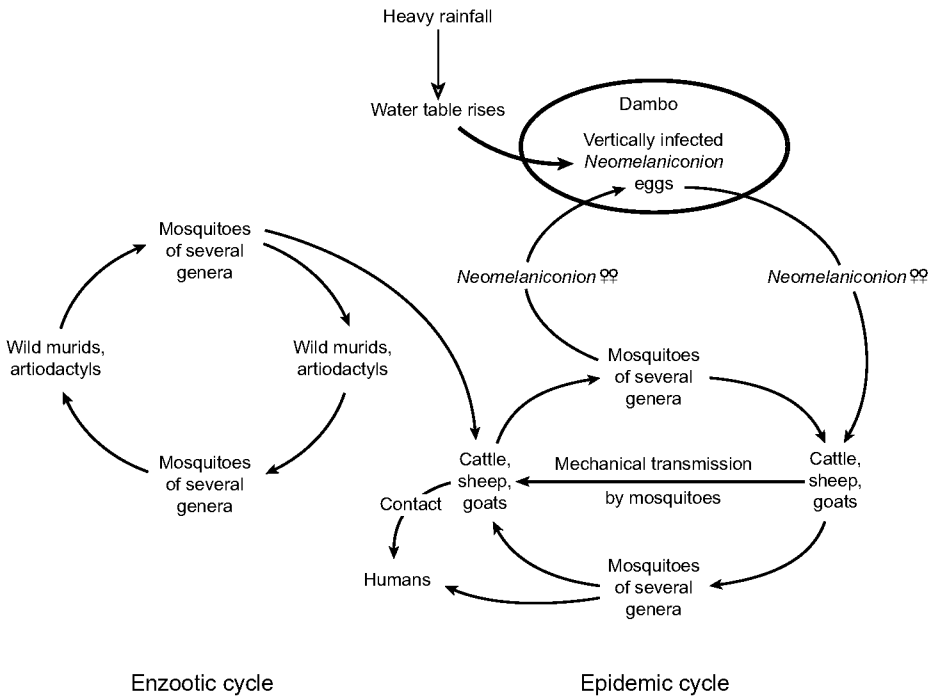


Figure 45.29 Hypothetical representation of the enzootic and epidemic transmission cycles of Rift Valley fever virus in eastern Africa. Enzootic cycles, which are inapparent, are thought to involve murine rodents and possibly wild artiodactyls as amplifying hosts. Through bridge vectors, RVFV may be transmitted to domesticated animals causing focal outbreaks of disease. Flooding of dambos by exceptionally heavy rain leads to the hatching of dormant *Neomelaniconion* eggs, then to the development of the aquatic stages and the emergence of huge numbers of adults, including a percentage of vertically infected females. Transmission of virus to farmed ruminants initiates an epidemic which is maintained by additional vector species. Transmission may involve species of *Aedes*, *Neomelaniconion*, *Culex* and *Ochlerotatus*. Humans may be infected by contact with the tissues of infected animals, or by inhalation of virus in droplets of blood. Arrows indicate the direction of virus transmission.

(a) Aquatic larval habitats of putative vectors

In the northern, Sahelian part of Senegal the climate is hot and dry, and the annual rainfall ranges from <300 to 500 mm. Here, large rain-filled, **temporary pools** spaced a few kilometres apart form a network of surface waters. The maximum surface area of the pools ranges from a few hundred square metres to several hectares. Areas of livestock pasture are interspersed with networks of temporary pools that can provide water for livestock and humans. During the long dry season, the densities of both livestock and mosquitoes are low, whereas during the short rainy season livestock are brought from elsewhere to graze near the rain-filled pools (Ba *et al.*, 2005). In contrast, where the Sénégal River forms the boundary between Mauritania and Senegal, the mosquito larval habitats are mostly **permanent pools** which favour the proliferation of *Cx. poicilipes* (Diallo *et al.*, 2005a). Dam construction on the Sénégal River may have caused the formation of extensive and long-lasting bodies of standing water (Jouan *et al.*, 1988; Walsh, 1988).

Most temporary pools are shallow, of 2 m depth at most, and because slopes of the ground surface are very gradual, small changes in water depth produce large changes in flooded surface area. Eggs of aedine mosquitoes are laid on moist soil at the edge of temporary pools, those with a highly varying water level being the most favourable. In

Table 45.29 Exposures during the previous 90 days to a variety of forms of contact with domesticated animals of human individuals of two classes: (i) recently infected with Rift Valley fever virus; and (ii) not infected with RVFV. (From Woods *et al.*, 2002.) The survey was undertaken in Garissa District, north-eastern Kenya, during 1997 and 1998.

Animal exposures	*Acute* [a] *infection (n = 31) (%)*	*No infection (n = 140) (%)*
Sheltered livestock in home after a flood	87	45
Killed an animal	64	34
Butchered an animal	45	24
Skinned an animal	65	27
Cooked meat	65	34
Milked animals	80	42
Drank raw animal milk	97	64
Care of animal during birth	68	33
Disposal of aborted fetus	61	26
Contact with sheep [b]	81	29
Contact with goats [b]	90	65
Contact with cows [b]	65	35
Contact with camels [b]	16	12

[a] Positive for RVFV-specific IgM.
[b] Contact included herding, cooking, slaughtering, contact with other body fluids, drinking raw milk.

the Ferlo area, a pastoral region, the rainy season lasts 4–5 months, from June or July to September or October. During the dry season, herds of cattle, sheep and goats concentrate around natural pools, the only places with free water, but when the rainy season starts, temporary pools refill and transhumant herds arrive. These pools are important watering places for livestock, and are the centres of ecosystems where vectors proliferate, notably *Am. vexans arabiensis* and *Cx. poicilipes*. The first rains do not always fill the pools because water is first absorbed by the sandy soil; >20 mm rainfall is needed to permit development of the aquatic stages. The aquatic stages of *Am. vexans arabiensis* develop so rapidly that adults emerge only 4 days after the first rains. Rain falls intermittently during the following months, until it eventually decreases and the pools dry up. *Aedimorphus vexans* is abundant at the beginning of the rainy season. Later, if a rainless period sufficiently long to permit the exposure of oviposition sites around the pools is followed by a period of heavy rainfall, another generation of substantial population density can arise during the same rainy season. Usually, about 2 months after the pools flood, the population density of aedine mosquitoes decreases while that of *Cx. poicilipes* and other culicines increases, reaching maximum abundance towards the end of the rainy season when the ground pools are covered by vegetation (Fontenille *et al.*, 1998; Chevalier *et al.*, 2004; Ba *et al.*, 2005; Mondet *et al.*, 2005).

(b) Adult biology of putative vectors

The Barkedji area in northern Senegal has a short rainy season, from mid-June to October, and an annual rainfall of 300–500 mm. Barkedji village is surrounded by a temporary pool that has a maximum surface area of several hectares. During the rainy season, when rain is intermittent, the pool periodically increases and shrinks in size. In most years, a single, early peak of abundance of *Am. vexans arabiensis* is followed by their disappearance. Enzootic cycles of RVFV in sheep were recorded in 1993 and 2002, years in which *Am. vexans* showed early and late peaks of population density. In 2002, RVFV was isolated from *Am. vexans* and *Cx. poicilipes* at the end of the rainy season. The investigators concluded that in this region the enzootiology of RVFV is explained by the timing of rainfall rather than by its quantity. In years when there is a single peak of *Am. vexans*, the population increases quickly but soon disappears. In years when rains resume after a substantial break, eggs laid earlier in the year hatch, as also do eggs from an earlier year that needed repeated flooding. *Aedimorphus vexans* can then act as a vector during a period when it is normally not abundant.

Sheep-baited traps were deployed at six locations on 25 trapping nights between mid-July and early-November 2002. *Aedimorphus vexans arabiensis* formed 88.1% of the catch, *Am. ochraceus* 3.1%, *Cx. poicilipes* 3.0%, and *Ne. mcintoshi* 0.5% (n = 8073).

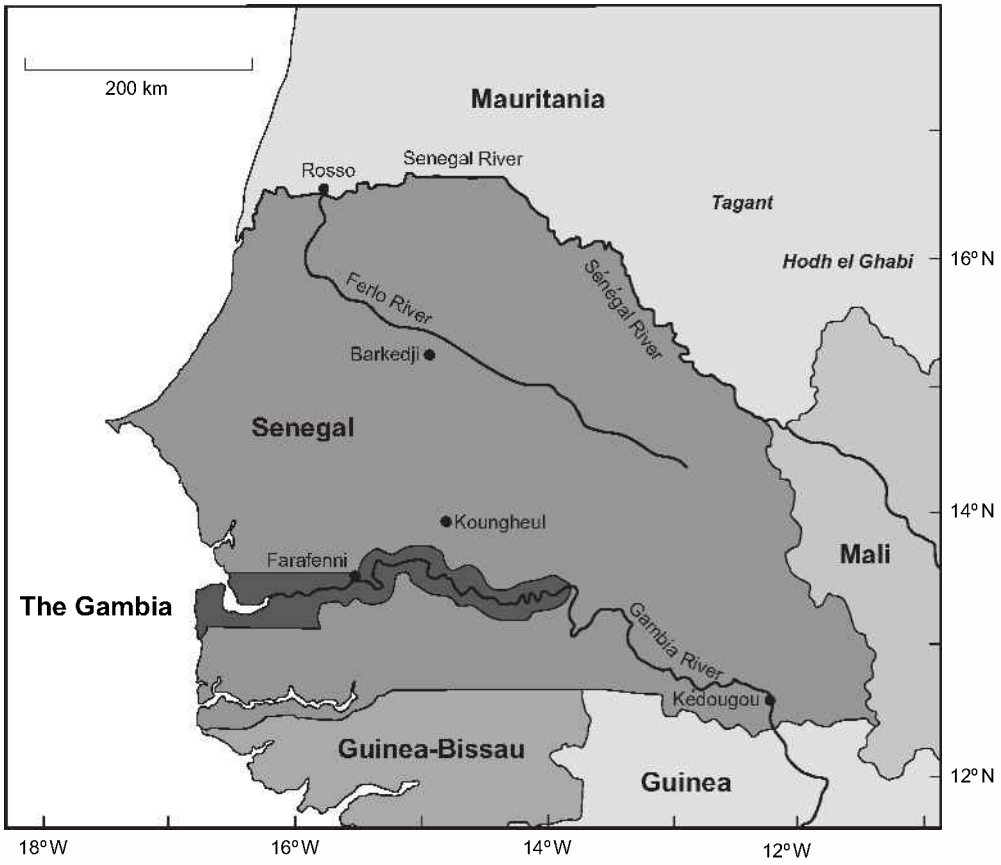


Figure 45.30 Map of Senegal, which surrounds The Gambia, and of parts of the adjacent countries – Mauritania, Mali, Guinea and Guinea-Bissau. The named localities and rivers are those referred to in the text.

In years with greater rainfall, the population density of *Cx. poicilipes* had been much greater. Chevalier *et al.* (2004) postulated that transhumance, the seasonal moving of herds of farmed animals, is a possible means of dispersion of RVFV.

From measurements of natural parity rates and mark–release–recapture studies, and with a number of assumptions, a daily survival rate of 0.95 was estimated for both *Am. vexans* and *Cx. poicilipes*, a value favourable for biological transmission. Estimated flight ranges for both species suggested that they would not carry the virus over long distances. Females of both species remained near the ground pool that was their larval habitat and did not move to nearby villages (Ba *et al.*, 2005).

(c) Enzootic transmission in West Africa

Virological and serological findings from a number of African countries indicate that amplifying hosts of RVFV may be found among a variety of wild mammals and among domesticated ruminants (Section 45.6.2.a), but only slender evidence is available from wild mammals in Central and West Africa. This includes a mean seropositivity rate of 0.7% in rodents (n = 287) in southern Mauritania (Saluzzo *et al.*, 1987), and specific antibodies found in low-to-moderate percentages in three rodent species in Senegal (Diop *et al.*, 2000). High seropositivity rates were recorded from game and other large mammals in the Central African

Republic and from bats in Guinea (Digoutte *et al.*, 1974a; Boiro *et al.*, 1987).

Very little is known of the vectors that transmit RVFV between wild mammals during inter-epizootic periods. In the Central African Republic, near the confluence of the Lobaye River with the Oubangui River (17° 43' E, 0° 30' S), RVFV (cited as Zinga virus) was isolated from *Mansonia africana*, *Neomelaniconion crassiforceps* and *Nemelaniconion jamoti* (Digoutte *et al.*, 1974a). The evidence is stronger for the putative vectors that feed on farmed animals. In the Sénégal River basin, RVFV was isolated from *Cx. poicilipes* (MIR, 2.5) (Diallo *et al.*, 2000). At Barkedji in northern Senegal, where circulation of RVFV had been detected, RVFV was isolated from *Am. vexans arabiensis* (MIRs, 0.54–2.41) and *Am. ochraceus* (MIRs, 1.4–2.4) (Fontenille *et al.*, 1995; Zeller *et al.*, 1997).

For a number of years after the major outbreak of RVF in the Sénégal River basin in 1978, herds of sheep or goats at locations in southern Mauritania and northern Senegal were serologically monitored for evidence of RVFV infections. Continuing cases of seropositivity in young animals in the absence of epizootics of RVF were taken to indicate enzootic transmission.

In mammalian hosts, the antibodies involved in most immune responses to arboviruses are IgM and IgG. IgM generally becomes detectable 1–2 days after the onset of clinical symptoms, but remains detectable for only 6–12 weeks. In contrast, specific IgG begins to increase in titre 1–2 weeks after the onset of symptoms, and remains detectable for many years, if not for life (Section 44.3.2). In Mauritania, at the end of the rainy season in October 1993, anti-RVFV IgM was present in herds at six out of seven locations (prevalence rates 2.8–43.5%), while anti-RVFV IgG was found at all seven locations (prevalence rates 1.5–54.3%). The presence of IgM in most herds showed continuing transmission (Zeller *et al.*, 1995). In Senegal, during the period 1992–1994, serological surveys were undertaken from locations in the Sénégal River basin in the north to Kédougou in the south. Anti-RVFV IgG was found in herds of cattle and sheep (prevalence rates 2.7–7.7%) but anti-RVFV IgM was not found (Zeller *et al.*, 1997). Together, these findings indicated that enzootic transmission cycles occur within herds of farmed animals in West Africa, and that domesticated ruminants are amplifying hosts of RVFV during inter-epizootic periods (Fontenille *et al.*, 1998; Chevalier *et al.*, 2004).

(d) Epizootics/epidemics in livestock and humans in West Africa

In mid-October 1987, at the end of the rainy season, an outbreak of RVF arose in the Sénégal River basin, and quickly developed in southern Mauritania and northern Senegal to a severe epizootic in domesticated ruminants and an epidemic in humans. In Mauritania, the first human case was hospitalized on 14 October, the peak of human cases occurred during early November, and the human epidemic ended by mid-December (Digoutte, 1999). Clinical, biological and epidemiological investigations, which were started immediately, revealed extensive amplification of the virus in domesticated ruminants (Digoutte and Peters, 1989; Knauert *et al.*, 1989). Herdsmen reported a high incidence of abortion and disease in their livestock. In one area, specific-antibody prevalence among livestock was as high as 85%, and approximately 80% of the sera were positive for both IgM and IgG, indicating recent onset of the disease. Sheep and goats predominated numerically over cattle, but the prevalence of specific antibodies was similar among all three species. The human population had specific-antibody prevalence rates of 40% or less, among which 90% were IgM positive. Analyses suggested that the risk factors for animals and humans were not equivalent (Ksiazek *et al.*, 1989).

This 1987 outbreak of RVF was the largest recorded in West Africa. In southern Mauritania the losses of livestock were great, and the number of human clinical cases was thought to be about 2300, with an estimated 225 deaths. In the hospital at Rosso, which is situated on the Sénégal River, a total of 284 human cases was identified, among whom the death rate was 9.9%. No significant

relationship was found between human infection with RVFV and animal ownership, occupation or sex, but there was a high correlation with drinking fresh milk. RVFV is not contained in milk, but drinking fresh milk involved regular contact with animals. There was no evidence of transmission from infected to uninfected patients in the hospital. Tests on serum samples (n = 659) from 12 villages showed that Hassophones had a significantly higher seropositivity rate than other groups of people; also, they appeared more often among hospital patients. In two villages, almost every human case of RVF was associated with animal abortion (Jouan *et al.*, 1988, 1989a; Digoutte, 1999). Less severe outbreaks of RVF were investigated elsewhere in the region. In Senegal, modest levels of transmission occurred along the Ferlo River, approximately 130 km south of the Sénégal River. In The Gambia, an outbreak of RVF among domesticated animals and humans occurred near Farafenni (Ksiazek *et al.*, 1989).

The 1987 epizootic occurred after a period of normal rainfall but at a time when mosquitoes were extremely abundant throughout the Sénégal River basin (Digoutte and Peters, 1989; Ksiazek *et al.*, 1989). It occurred after the construction of a dam at Diama, which led to a great increase in standing water, but causality could not be proved (Walsh, 1988). Comparisons of incidence between adjacent encampments or adjacent villages suggested that ecological conditions that produce dense vector populations were necessary for initiating epizootics in domesticated animals, which could lead to epidemics in human populations. Transmission from animals to humans appeared not to be predominantly by mosquito bite but rather due to close animal–human interactions, e.g. through ritual slaughter and association with animal abortions (Jouan *et al.*, 1989a).

An outbreak of RVF occurred during 1988 in the Hodh el Gharbi region of Mauritania, near its border with Senegal (Figure 45.30). During the following 2 years, adult mosquitoes were captured in light traps in areas close to aquatic habitats at seven locations in the outbreak area, and on the Senegal side of the border at five locations which were on pathways of livestock exchange between the two countries. A total of 92,787 mosquitoes of ten genera and 41 species were captured. In south-eastern Mauritania, where the aquatic habitats were temporary ground pools, the most abundant species were *Am. vexans* (77%) and *Cx. poicilipes* (15%). In Senegal, where the aquatic habitats were riverine pools and more permanent pools (habitats not favoured by floodwater aedines), the most abundant species were *Cx. poicilipes* (41%) and *Mansonia uniformis* (39%). RVFV was isolated only from *Cx. poicilipes*; from 36 pools in Senegal in 1998, and from 27 pools in Mauritania in 1999. Although RVFV was not isolated from *Am. vexans*, Diallo *et al.* (2005a) surmised that *Am. vexans* might facilitate emergence of RVFV while it was present during the early rains, and that *Cx. poicilipes* is the main vector during the late season.

45.6.6 Transmission of RVFV in Egypt

The northwards dispersion of Rift Valley fever virus led to outbreaks of RVF in Egypt and some years later in the Arabian peninsula where, during 2000, infections in livestock and humans occurred in Yemen and Saudi Arabia (WHO, 2000b; Jupp *et al.*, 2002).

Substantial information has been gained on RVFV transmission in Egypt. Over the Nile Valley and Delta there is no rain but irrigation water is abundant, so water-filled canals and ditches and bodies of waste water are common, and provide aquatic habitats for the putative vectors of RVFV. The role of irrigation water in enabling the build-up of massive mosquito populations was demonstrated in a neighbouring country with a similar climate. In the Jizan region of Saudi Arabia, where RVFV was isolated from *Am. vexans arabiensis* and *Cx. tritaeniorhynchus*, adults of those species were trapped in large numbers near a variety of aquatic habitats. Near flooded agricultural fields, 800 *Cx. tritaeniorhynchus* and *Am. vexans* were captured per trap hour in light traps. At a sedge bed at the periphery of a large dam, 18,600 *Cx. tritaeniorhynchus* were captured per trap hour in net traps. *Aedimorphus vexans* was more common near

temporarily flooded fields, whereas *Cx. tritaeniorhynchus* was more prevalent near permanent or semi-permanent aquatic habitats (Jupp *et al.*, 2002).

The human population density in the Nile Valley and Delta is among the highest of any region of the world. Sheep and goats are ubiquitous in the irrigated fields and in the hamlets and villages. Donkeys, cattle, buffalo, camels and dogs are as widely distributed but less numerous. The outbreaks of Rift Valley fever in Egypt in 1977 and 1978, with many thousands of human cases and almost 600 recorded deaths, stimulated detailed epidemiological investigations, partly undertaken by a US Naval Medical Research Unit (Hoogstraal *et al.*, 1979; Meegan, 1979; Meegan *et al.*, 1980).

Four murine rodents (*Arvicanthis niloticus*, *Acomy cahirinus*, *Rattus rattus* and *Mus musculus*) were common in fields and villages throughout the Nile Valley and Delta, and constituted the major part of the wild mammal fauna. Anti-RVFV HI antibodies were found in the first three of those species; however, when inoculated with RVFV, only low-magnitude viraemias of short duration developed, and these species were thought not to serve as amplifying hosts (Hoogstraal *et al.*, 1979). RVFV was isolated from the brain of a *Rattus rattus* (cited as *R. r. frugivorus*) (Imam *et al.*, 1979). During the 1977 epizootic, blood samples for virological analysis were taken from domesticated animals on farms in areas where deaths and/or abortions occurred; RVFV was isolated from sheep (27/58), a goat (1/9), a cow (1/17), a horse, (1/18) and a camel (1/30) (Imam *et al.*, 1979).

In two investigations conducted in the Nile Delta and Valley, *Culex pipiens* was found to be the dominant mosquito species, and in one collection they formed 94% of the catch (n = 41,675). Other species occurred in only limited numbers. In the field, *Cx. pipiens* fed readily on sheep and humans (Hoogstraal *et al.*, 1979). In another collection, *Cx. pipiens* formed >95% of the catch (n = 55,126) (Meegan *et al.*, 1980). From collections made in villages after the peak of disease incidence, RVFV was isolated from two pools of *Cx. pipiens* (MIR = 0.05, n = 38,538). *Culex pipiens* could transmit RVFV in the laboratory. Females of an F_4 generation from the wild that fed on a hamster with a viraemia of 10^6 SMIC LD_{50} ml^{-1} transmitted virus to uninfected hamsters when tested on days 12 to 36 after the infective feed (Meegan *et al.*, 1980). A different situation was found in studies undertaken in the Aswan Governorate in southern Egypt after an outbreak of RVF there in 1993. Of the mosquitoes captured in a local village, 94% were *Oc. caspius*. The females attacked both sheep and humans, and laboratory tests showed *Oc. caspius* to be the most efficient vector of RVFV among the Egyptian mosquitoes tested. *Culex pipiens* and *Cx. antennatus* were named as other 'potential vectors' (Turell *et al.*, 1996).

The 1977 epidemic of Rift Valley fever in Egypt was unique in the extent of human infection that occurred. Transmission by vectors was considered important, although it had not been quantified, but a high proportion of the human infections were ascribed to inhalation of virus in blood droplets discharged during the slaughter of sheep by the traditional method of throat cutting (Section 44.4.4).

45.6.7 Summary

Rift Valley fever virus is widely distributed in mainland Africa. It is present also in Madagascar, and recently has spread to the Arabian peninsula. The virus infects wild populations of a wide variety of mammal species, but which are amplifying hosts is not known. It readily causes fatal infections in young domesticated ruminants, with serious financial consequences. The characteristics of the transmission cycle vary between geographical regions. Major outbreaks of disease are spasmodic, and require ecological conditions that permit the production of massive populations of vectors. In eastern and southern Africa, enzootics/epidemics occur only during years of exceptionally heavy rainfall when a rise of the water table fills dambos and pans. In these regions, the putative main vectors are species of *Neomelaniconion*, and vertical transmission may enable RVFV to survive through long dry inter-epizootic periods within the dormant

eggs. In West Africa, major outbreaks are not associated with exceptionally heavy rainfall. In some parts of this region the aquatic habitats are very large seasonally filled pools, and in other parts they are permanent pools. The putative main vectors are species of *Aedimorphus* and *Culex*. In Egypt and the Arabian peninsula, water from irrigation schemes provides larval habitats for the putative vectors, which are species of *Culex* and *Ochlerotatus*.

Knowledge of the transmission cycles (Figure 45.29) is based largely on circumstantial evidence. The wild amplifying hosts of RVFV have not been identified with certainty, but possibly include murid rodents and artiodactyls. During outbreaks in all regions, young domesticated ruminants (lambs, goat kids, calves) are the principal amplifying hosts. The extent to which domesticated ruminants serve as amplifying hosts during inter-epizootic periods is uncertain. Species of *Aedimorphus*, *Neomelaniconion*, *Ochlerotatus* and *Culex* are natural hosts of RVFV, and some are putative vectors. The main and subsidiary vectors vary with geographical region. In eastern Africa, outbreaks are associated with the emergence from newly flooded dambos of massive numbers of *Ne. mcintoshi*, and the first infections may result from the bites of vertically infected females. Later, other vector species make use of the same dambos and maintain virus transmission. There is insufficient information to indicate whether or not vertical transmission is an important factor in maintaining transmission. The incidence of Rift Valley fever among humans varies between countries. There is little evidence of transmission to humans by the bite, but close contact with infective animals presents grave risks of infection. The virus may be carried as an aerosol during certain forms of slaughter, or may be transferred by physical contact with aborting females or with the organs of diseased animals at slaughter or post-mortem examination.

45.7 TRANSMISSION OF WEST NILE VIRUS

45.7.1 Introduction

West Nile virus (WNV) is a member of the Japanese encephalitis virus serogroup of the genus *Flavivirus*, family *Flaviviridae*. The characteristics of the genus *Flavivirus* are described in Section 44.1.2.a. WNV is one of the eight viruses that constitute the JEV serogroup, one of the seven of which the principal amplifying hosts are birds, and one of the six that are pathogenic in humans (Blitvich, 2008). It is the pathogenic agent that causes human West Nile fever (WNF), an emerging disease for which, despite extensive research, some aspects of its epidemiology remain speculative. The pathology of West Nile virus in humans and certain other vertebrates is described in Section 44.10.2.d.

West Nile virus was first detected in Uganda in 1937, in a serum sample taken from a febrile patient. A broader survey undertaken in 1939 located human cases in the Sudan, Uganda, Kenya and the Congo, where it appeared to cause only silent infections. As described in detail below, WNV was transported from that region, probably by birds, to the Middle East and North Africa, and from the Middle East to Central and Eastern Europe and, eventually, to the New World. At intervals, epidemics of WNF occurred in human populations, and in locations where the more virulent strains of WNV were present they could be of great severity. Enzootics have been recorded in populations of wild birds and in flocks of domesticated birds. West Nile virus must have been enzootic in birds in the African tropics since long before its discovery in a human case in Uganda in 1937. Subsequent to that, outbreaks of WNF were reported from many countries.

45.7.2 Isolates, strains, clades and lineages of West Nile virus

Being RNA viruses, arboviruses have very high mutation rates and so a potential for rapid change. The high mutation rates result partly from lack of the proofreading enzymes that would assure fidelity of replication. Further, the high mutation rates are coupled with high rates of replication, a combination that is reflected in rates of RNA genome evolution that exceed the rates of DNA chromosome evolution of their hosts by $>10^6$–fold. As a result, the concept of 'wild type' often has only a short-term relevance for RNA genomes (Holland *et al.*, 1982). Even so, arboviruses can have a marked genetic stability, and at any one time more than one form of a virus may be circulating within a small geographical region.

(a) Isolates and strains

In epidemiological investigations of arboviruses, it can be important to know the precise identity in genomic terms of the forms of an arbovirus that are circulating, and that requirement can be met by use of a formal system for the characterization and designation of isolates. West Nile virus is exceptional in the publication of hundreds of named isolates as an outcome of research. In all cases **isolates** are given a name, with associated details of the host species or taxon from which they were collected, the year of collection, and the site of collection – whether as city, county, state or country (Table 45.30). Molecular-genetic details of the isolate will have been obtained, usually the nucleotide sequences of a portion or of the whole

of the genome. To register a new isolate, the nucleotide sequence data are sent to GenBank, a part of the International Nucleotide Sequence Database Collaboration, and, if accepted, the isolate is allocated a GenBank Accession number – the key form of identification because isolate names can be very similar. Knowledge of the accession number of any viral isolate provides for access to details of its genomic structure, from which its relationship to other isolates can be determined.

Separate isolates of WNV may differ only slightly genomically, and in such cases those isolates, however widely distributed, constitute a **strain**. The occurrence of genomic changes that are sufficient to define a new, distinctive genotype heralds the appearance of a new strain, which in a phylogenetic tree composed of sufficient isolates to reveal evolutionary relationships would be apparent as a new lineage. The phenotypic characteristics of the new strain might or might not be readily apparent, but some new strains are of epidemiological importance. Unfortunately, few investigations produce sufficient genomic data to group isolates into strains. Charrel *et al.* (2003) commented that the failure to describe the complete sequences of a sufficient number of strains of WNV, especially in the Old World, made it difficult to clearly elucidate the evolutionary relationships of many strains. Accepting that determination of the full genomic sequences of many isolates was impractical for the purpose of generating reliable phylogenies, they established that co-linearized *E–NS3–NS5* gene sequences provide a 'valuable surrogate' for complete sequences. (The use of partial sequences of the NS3, NS5 and E protein genes is described briefly in Section 44.1.2.b.)

From 1999 to 2001, isolates of WNV collected in and near New York were genomically relatively homogeneous and were designated the NY99 strain, of which the isolate NY99-flamingo was specified as the prototype. During 2002–2003, another well-supported strain, WN02, became distinguishable, and by 2004 it had disseminated through all continental states of the USA, becoming epizootically dominant (Ebel *et al.*, 2004). Different authors used the descriptive terms '**clade**' or '**genotype**' for those two groups of isolates. Both are appropriate, but the term 'clade' implies a known position on one or more phylogenetic trees, whereas the term 'genotype' is much less explicit.

(b) Lineages

From a phylogenetic tree generated from nucleotide sequences of isolates of WNV from Africa, the Middle East and Europe, Berthet *et al.* (1997) observed that the strains formed two distinct lineages, each of closely related subtypes present in large and overlapping geographical areas. They distinguished these as lineages 1 and 2. This grouping was generally accepted, and Figure 45.31 shows one of a number of phylogenetic trees later produced. Subsequently, isolates of WNV were collected that showed greater genetic diversity and evolutionary divergence, and additional numbered lineages were proposed for them (Table 45.30).

Lineage 1. Phylogenetic trees constructed from the aligned nucleotide sequences of WNV strains showed strains from Senegal, the Central African Republic, Kenya, Egypt, Israel, Europe, Russia, Australia and the north-eastern USA forming a major clade, which was designated lineage 1. That could be subdivided into three monophyletic subsidiary clades: clade 1a strains were widely distributed across the regions and countries listed above; clade 1b was of related isolates from Australasia which had at one time been named Kunjin virus; a clade 1c designated for certain strains from India was no longer recognized after their transfer to the later designated Lineage 5 (Berthet *et al.*, 1997; Lanciotti *et al.*, 2002; Bondre *et al.*, 2007).

Kunjin virus, which is present in all mainland states of Australia, in New Guinea, Sarawak and other parts of Borneo, was once considered a distinct flavivirus species. Sequence analysis of isolates from different parts of Australia showed them to form a closely related group which could be differentiated into subgroups, and to be distinct from all other forms of WNV. Kunjin virus was first isolated in 1960, in Northern Queensland, from *Cx. annulirostris* its main vector in Australia, where the amplifying hosts are ciconiiform birds, particularly the rufous night heron. Kunjin virus is

Table 45.30 Lineages and other details of isolates of West Nile virus that either are mentioned in the text or are illustrative of isolate citations.

Isolate name	*GenBank accession no.*	*Source*	*Year*	*Host from which isolated*	*Refs*
Lineage 1, 1a					
Egypt 101	AF260968	Egypt	1951	Human	4, 7
Maayan Zvi	–	Israel	1951	Human	13
goose97	AF380663	Israel	1997	Domesticated goose	15
goose98	AY033388	Israel	1998	Domesticated goose	5
goose99	AY033391	Israel	1998	Domesticated goose	5
WN-Israel 1998	AF205882	Israel	1998	Domesticated goose	1, 18
GooseKY98	AY052412	Israel	1998	Domesticated goose	14
GooseMaV00	AF380666	Israel	2000	Domesticated goose	14
Israel 1998-stork	AY033389	Israel	1998	*Ciconia ciconia ciconia* *	4
stork-1998 IS98-STI	AF481864	Israel	1998	*Ciconia ciconia ciconia*	5, 7
gull99	AY033390	Israel	1999	*Ichthyaetus leucophthalmus* ⊕	5
WN-0043	AF375042	Israel	2000	Human	16
WN-0233	AF375043	Israel	2000	Human	16
WN-0247	AF375044	Israel	2000	Human	16
WN-0304	AF375045	Israel	2000	Human	16
ALG-ArDjanet	AF001567	Algeria	1968	*Culex* sp.	2, 15
PaH001	AY268133	Tunisia	1997	Human	7
KN3829	AF146082	Kenya	1998	*Culex univittatus*	7
CAR-HB6343	AF001558	Central African Republic	1989	Human	2, 15
SEN-ArD93548	AF001570	Senegal	1993	*Culex neavei*	2, 15
RO96-1030	AF130363	Romania	1996	Human	2
RO97-50	AF260969	Romania	1996	*Culex pipiens*	4
RO97-50mos	AF130362	Romania	1997	Mosquito	7
Russia-1999	AF317203	Volgograd	1999	Human	16
PaAn981	AF205883	Italy	1998	Horse	18
NY99-flamingo § ≠	AF196835	New York	1999	*Phoenicopterus chilensis* †	1, 4
NY99-equine ≠	AF260967	New York	1999	Horse	4, 7
NY1999-human ≠	AF202541	New York	1999	Human	4, 7
Conn-1999	AF206518	Connecticut	1999	*Culex pipiens*	16
MD2000-crow265	AF404753	Maryland	2000	*Corvus brachyrhynchos* ‡	4, 7
02003555 #	AY369416	Onondaga, New York	2002	*Corvus brachyrhynchos*	12
007365 #	AY369418	Schenectady, New York	2002	Horse	12
Alabama-1	AY428523	Alabama	2001	*Culex quinquefasciatus*	19
Alabama-2	AY428524	Alabama	2001	*Corvus brachyrhynchos*	19
Mosq. V4369	AY712948	Harris Co., Texas	2003	*Culex quinquefasciatus*	17
AZ 2004	DQ164201	Arizona	2004	Human	17
Bird 9483	DQ158243	Galveston Co., Texas	2004	*Cyanocitta cristata* ω	17

Continued

Table 45.30 Continued.

Isolate name	*GenBank accession no.*	*Source*	*Year*	*Host from which isolated*	*Refs*
Lineage 1, 1b					
KUNMRM 16	AF196505	Australia¶	1960	*Culex annulirostris*	3
KUNMP502-66	AF196534	Sarawak, Borneo	1966	*Culex pseudovishnui*	3
KUN35911	AF196511	New South Wales, Australia	1984	Horse	3
Lineage 2					
B956	AY532665	Uganda	1937	Human	10
WNFCG	M12294	Uganda	1937	Human	4, 7
MP22	AF001562	Uganda	1959	*Coquilletidia metallica*	2, 4, 15
WNFCG	M12294	Uganda	1937	Human	4, 7
B956	AY532665	Uganda	1937	Human	10
Madagascar-ArMg7	AF001574	Madagascar	1978	*Culex univittatus*	6, 15
Lineage 3					
RabV	AY765251	Czech Republic	1997	*Culex pipiens*	8
Lineage 4					
LEIV-kmd88-190	AY277251	Caucasus	1998	*Dermacentor* sp.	8
HU2925/06	GU047875	Spain	2006	*Culex pipiens*	11
Lineage 5					
India804994	DQ256376	Southern India	1980	Human	4, 9
WNIG2267Cv	AY944243	Southern India	1955	*Culex vishnui*	9
WN821622H	AY944247	Southern India	1982	Human	9

*, White stork; ⊕, White-eyed gull; §, Prototype New York strain; ≠, NY99 clade; †, Chilean flamingo; ‡, American crow; #, WN02 clade; ω, Blue jay; ¶, Cape York Peninsula.
References: 1, Lanciotti *et al.* (1999); 2, Savage *et al.* (1999); 3, Scherret *et al.* (2001); 4, Lanciotti *et al.* (2002); 5, Malkinson *et al.* (2002); 6, Burt *et al.* (2002); 7, Charrel *et al.* (2003); 8, Bakonyi *et al.* (2005); 9, Bondre *et al.* (2007); 10, Iyer *et al.* (2009); 11, Vázquez *et al.* (2010); 12, Ebel *et al.* (2004); 13, Goldblum *et al.* (1954); 14, Banet-Noach *et al.* (2003); 15, Berthet *et al.* (1997); 16, Hindiyeh *et al.* (2001); 17, Davis *et al.* (2005); 18, Autorino *et al.* (2002); 19, Davis *et al.* (2003).

a minor cause of human arboviral encephalitis in Australia (Mackenzie, 2001; Scherret *et al.*, 2001). When and how this distinct form of WNV reached Australasia is enigmatic.

Lineage 2 strains are endemic to sub-Saharan Africa and Madagascar. Strains have been isolated from west, central and east Africa and from Madagascar. Transmission cycles are enzootic, and humans appear to be dead-end hosts (Savage *et al.*, 1999; Lanciotti *et al.*, 2002). Experimental and observational findings indicated that highly neuroinvasive and less neuroinvasive phenotypes exist in strains of both lineage 1 and lineage 2 (Botha *et al.*, 2008).

Lineage 3 was proposed by Bakonyi *et al.* (2005) for the strain RabV, which had been isolated in 1997 from mosquitoes at Rabensburg in the Czech Republic. An analysis by Brault (2009) of the complete genomes of 20 strains of WNV supported the assignment of RabV to this new lineage.

Lineage 4 was proposed by Bakonyi *et al.* (2005) for the strain LEIV-Kmd88-190 (= Rus98) which was isolated in 1988 in the Russian Caucasus from the ixodid tick *Dermacentor marginatus*. Brault's (2009) analysis of 20 strains of WNV supported the assignment of LEIV-Kmd88-190 to this new lineage. Sampling for flavivirus infections in Spanish mosquitoes yielded a single pool of 50 unfed *Cx. pipiens* complex females (pool HU2925/06), captured in 2006 at Palos de la Frontera. Phylogenetic analysis of this and other WNV strains aligned them with 99% certainty

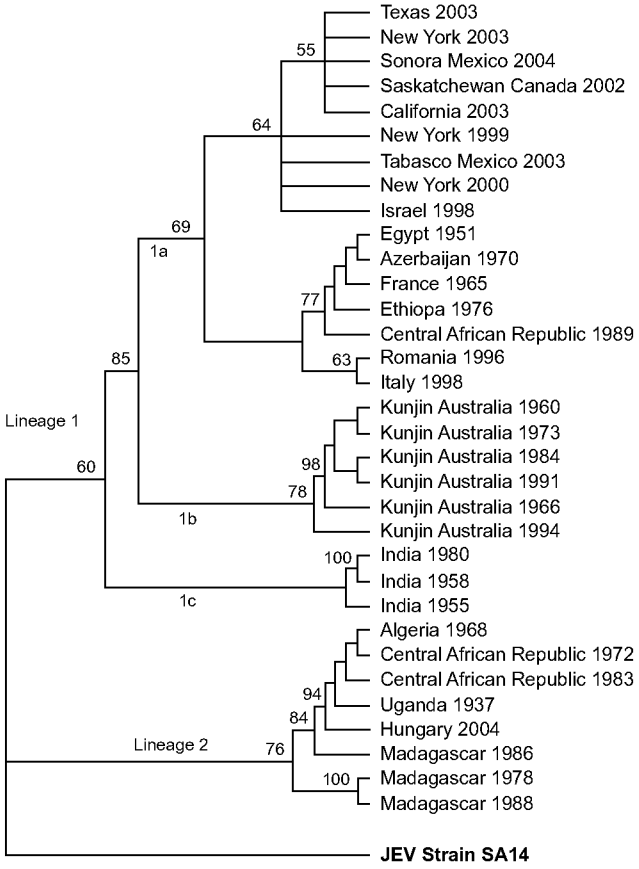


Figure 45.31 Phylogenetic tree of 33 strains of West Nile virus from different geographical regions. (From Blitvich, 2008; with the permission of Cambridge University Press.) The tree was generated by parsimony analysis of aligned nucleotide sequences in a 255-bp region of the enveloped gene. It is rooted using Japanese encephalitis virus, strain SA14, as an outgroup. The values above some branches represent percentage support by parsimony bootstrap analysis.

with LEIV-Kmd88 of lineage 4 (Vázquez *et al.*, 2010).

Lineage 5. Among many virus isolates that had been maintained at the lowest passage level were 15 of WNV, collected over a 27-year period, from a range of hosts and from different localities in southern India. Phylogenetic analyses of those isolates revealed that two, dating from 1967–1968 and isolated from a human and a fruit bat, were lineage 1 strains. The other 13 isolates, collected between 1955 and 1982, formed a distinct new lineage that was numbered 5. The 13 hosts were humans (2), *Culex* (*Culex*) *vishnui* (6), three other species of *Culex* (*Culex*) (4), and *Anopheles subpictus* (1) (Bondre *et al.*, 2007).

It is likely that further lineages of WNV will be designated. Mackenzie and Williams (2009) suggested that the Sarawak strain of Kunjin virus be ranked as a new lineage, and that Koutango virus [AF013360] be considered a further new lineage of WNV.

45.7.3 West Nile virus in the Old World

(a) First discoveries

During 1937, as part of an epidemiological investigation by the Yellow Fever Research Institute at Entebbe, Uganda, blood samples were taken from many people suffering from an illness suggestive of yellow fever or from a fever of unknown cause, and

were screened by intracerebral inoculation into mice. A neurotropic virus isolated from one blood sample was pathogenic to mice, and induced encephalitis and death when inoculated intracerebrally into rhesus monkeys. Lesions were limited to the central nervous system, and differed from those induced in humans by other neurotropic viruses. Serologically, the virus was related to 'Japanese B encephalitis virus'. The blood sample had been taken from a febrile, 37-year-old African woman in Omogo (1° 26' N, 33° 49' E) in the West Nile district of the Northern Province of Uganda, and was named West Nile virus. Serum obtained from one of five individuals living elsewhere in the West Nile district contained anti-WNV neutralizing antibodies (Smithburn *et al.*, 1940).

A survey of sera from 1428 individuals carried out in 1939 revealed that 16% of residents in the West Nile district of Uganda were positive for anti-WNV neutralizing antibody, and that WNV had been active also in the Sudan, Kenya and Belgian Congo (Smithburn and Jacobs, 1942). Later, screening human blood samples taken during the years 1950 to 1952 showed WNV to have been endemic not only in West Nile Province but also in the southern Sudan, and along the Nile valley into Egypt (Smithburn, 1952; Goldblum *et al.*, 1954). Neutralization tests revealed immunity to WNV in localities widely scattered through Egypt, with mean positivity rates of 54.9% in children (n = 142) and 76.2% in adults (n = 144) (Smithburn *et al.*, 1954).

An early investigation in the Nile Delta of Egypt, which revealed the role of certain birds as amplifying hosts of WNV and the role of *Culex* species as its vectors, had the breadth and quality to be a model for later epidemiological studies. During 1953, a survey was undertaken of indigenous birds that were abundant enough to produce a large crop of non-immune juveniles each year. In the region of Sindbis, an area of WNF endemicity, wild-caught birds of all common species had WNV-specific neutralizing antibodies (Table 45.31). In experimental studies, birds of five species that were shown to be free of WNV-specific neutralizing antibodies and that were exposed to the bites of WNV-infective mosquitoes became viraemic, developing titres of up to 9.5 (reciprocal log of titre). During the winter season, the seropositivity rate among hooded crows was 88%, but in late spring it fell to 40%, probably as a result of the appearance of non-immune fledglings. Death rates were very high among the hooded crows (*Corvus corvix*) and house sparrows (*Passer domesticus*), but most deaths did not occur until the fourth day post-infection, when the birds had been viraemic for 3 days (Work *et al.*, 1955).

Table 45.31 Findings from field and laboratory investigations on native birds exposed to West Nile virus in the Nile Delta region of Egypt. Seropositivity rates were determined in birds wild-caught around Sindbis. Viraemia titres and durations were measured in birds that had been exposed to infective mosquitoes, the titres being measured by intracerebral inoculation of serial dilutions of the avian blood into mice. (From the data of Work *et al.*, 1955.)

Species	*Seropositivity rate at Sindbis* *		*Upper viraemia titres (range)*	*Viraemias > 3.5 on days post-infection*	*Death rate*	*Transmission from Culex spp. to mice* †	
	(%)	(n)	*(reciprocal log)*	*(days)*		*No. bitten*	*No. infected*
House sparrow ‡	57	35	>3.5–8.0	1 to 4	12/16	25	23
Hooded crow §	88	17	>3.5–9.5	1 to 4	13/13	14	13
Laughing dove ¶‡	48	33	3.5–3.6	day 1 only	0/4	3	0
Common kestrel ≠	100	6	3.5–4.9	3 to 4	0/7	6	9
Cattle egret #‡	68	28	>3.5–3.8	day 1 only	0/5	3	3

*, Positivity for WNV-specific neutralizing antibodies during the winter season. †, Females of *Cx. pipiens*, *Cx. perexiguus* and *Cx. antennatus* that had become infective with WNV after ingesting infectious blood from naturally infected birds were later fed on mice, and the numbers of mice that became infected were recorded. ‡, *Passer domesticus*. §, *Corvus corvix*. ¶, Named palm dove (*Streptopelia senegalensis*) by these authors. ≠, *Falco tinnunculus*. #, Named buff-backed heron (*Bubulcus ibis*) by these authors. The scientific names given accord with 'Avibase – the world bird database', at http://avibase.bsc-eoc.org/.

WNV was isolated from wild-caught *Cx. pipiens*, *Cx. perexiguus* (cited as *Cx. univittatus*) and *Cx antennatus*, all of which fed readily on wild birds. In the laboratory, females of all three species became infected upon feeding on birds viraemic with WNV, and later those mosquitoes transmitted WNV when they fed on uninfected birds. In that way the mosquitoes showed vectorial competence. Females of *Cx. pipiens* and *Cx. perexiguus* transmitted WNV to mice after feeding on bird blood with titres as low as 3.5. Of five bird species exposed to infective mosquitoes, the viraemias that developed in the house sparrow, hooded crow (*Corvus corvix*) and common kestrel (*Falco tinnunculus*) were of sufficient titre to infect other blood-feeding mosquitoes, whereas the titres recorded in laughing dove (*Streptopelia senegalensis*) and cattle egret (*Bubulcus ibis*) were borderline (Work *et al.*, 1955).

Serological testing of human populations carried out in Egypt during 1952–1954 revealed country-wide seropositivity for WNV, some positive cases undoubtedly persisting from earlier infections. No suspected epidemics were reported during the surveys, but the results suggested that WNF was endemic in Egypt (Smithburn *et al.*, 1954). Intensive studies in the Nile Delta showed WNV infection to be essentially a disease of childhood, self-limited, non-fatal, and only rarely with manifestations of encephalitis (Taylor *et al.*, 1956).

(b) Epidemics of West Nile fever in the Old World

In **Israel**, localized outbreaks of an acute, infectious and self-limiting disease occurred during 1951 and 1952. WNV was isolated for the first time in 1951 from a sick child in Maayan Zvi, a communal settlement near Haifa, where the familiarity of local physicians with the syndrome suggested that it had occurred there over a number of years. The disease was named West Nile fever (Bernkopf *et al.*, 1953; Goldblum *et al.*, 1954). Further, mostly localized, outbreaks of WNF of up to 400 cases occurred in Israel until the mid-1950s, with no deaths; however, in 1957, among a total of 320 confirmed cases of WNF there were four fatalities within a group of 26 elderly patients (Spigland *et al.*, 1958). The following four decades saw only a small outbreak in the Negev desert in 1980, with no fatalities. In none of those outbreaks was virulence severe.

During 1998, a highly virulent outbreak of WNV occurred in farmed geese (Subsection 47.7.3.c below), and in 1999 a survey revealed seropositivity among 85% of people who had close contact with geese. In 2000, a country-wide outbreak of WNF among humans was characterized by marked virulence. It started in mid-August in central Israel, from where it spread north and south, peaking in September and declining in October. From mid-August until the end of October 2000, high morbidity was recorded throughout the country. The total number of confirmed cases was 439, with 29 fatalities. Of hospitalized patients, 73% had some form of CNS involvement. Incidence per 1000 population increased from 0.01 in the first decade of life to 0.87 in the ninth decade. Genomic analysis of four isolates revealed two distinct lineages: (i) a lineage of two isolates (WN 0233 and 0304, both Israel 2000) with 98% homology to a mosquito isolate from Romania 1997 and a human isolate from Volgograd 1999; and (ii) another lineage of two isolates (WN 0043 and 0247, both Israel 2000) with 99.7% homology to flamingo, mosquito and horse isolates from New York 1999 (Bin *et al.*, 2001; Weinberger *et al.*, 2001).

The characteristics of outbreaks of WNF in **Europe** were summarized by Hubálek (2000). Natural foci of enzootic WNV involving an avian–mosquito cycle, with predominantly passerine birds and species of *Culex*, were present in many wetlands in climatically warm and some temperate parts of Europe. Epidemics in human populations usually burst out with full strength in the first year, while during the following 1–2 years few cases were observed. Smaller epidemics or clusters of cases lasted for only one season. The outbreaks were associated with high populations of *Culex* species, caused by flooding and subsequent dry, warm weather, or with the formation of suitable larval habitats. Urban outbreaks involving the molestus ecotype of *Cx. pipiens* could be very serious, as in Bucharest in 1996.

Seroprevalence data indicated WNV activity in southern Romania during the 1960s or earlier. During 1996, the first major epidemic of West Nile disease in Europe occurred in the plain of the Danube Valley, and most prominently in the city of Bucharest. Of 767 patients with characteristic symptoms, laboratory tests confirmed infection with WNV in 393, all with neurological disorders: encephalitis 16%, meningitis 40%, meningo-encephalitis 44%. The illness progressed to coma in 13% of cases and resulted in 17 deaths. The actual case rate and death rate may have been much higher because national surveillance was delayed and relatively few clinical samples were tested. Serosurveys conducted during the epidemic pointed to a recent introduction of WNV to Bucharest (Tsai *et al.*, 1998; Campbell *et al.*, 2001).

Following the first isolation of WNV north of the drainage basin of the Caspian Sea in the 1960s, sporadic, small outbreaks of West Nile fever were recognized in southern regions of the then Soviet Union (Lvov *et al.*, 2004). In 1999, a widespread outbreak of WNF occurred in **southern Russia**, in the regions of Volgograd (48° 43' N, 44° 25' E) and Astrakhan (46° 21' N, 48° 01' E), both within the drainage basin of the Caspian Sea but in ecologically different deltas, and in the region of Krasnodar (45° 43' N, 48° 43' N) north of the Black Sea. Laboratory-verified cases exceeded 500, but through serological screening before and after the outbreak the number of cases was estimated to have exceeded 200,000. The death rate was about 10% (Lvov *et al.*, 2000, 2004). The clinical features of the Volgograd epidemic differed from those of previous outbreaks in their greater severity and higher death rate. Acute aseptic meningitis or encephalitis were frequently observed, and of 84 such cases 40 were fatal (Platonov *et al.*, 2001).

An outbreak of aseptic meningitis and encephalitis occurred in the Sfax and Mahdia coastal districts of **Tunisia** in the autumn of 1997. From a total of 173 hospitalized cases, infection with WNV was demonstrated in 86% of patients from whom samples were taken; there were eight fatalities (Triki *et al.*, 2001; Riabi *et al.*, 2010). Sequence analysis revealed that a human isolate, PaH001, collected during the Tunisian outbreak, was the closest relative of a virus collected in Israel (stork-1998 IS98-STI) and a group of highly virulent viruses that spread into North America (including NY1999-human, NY99-equine and NY99-flamingo) (cf. Table 45.30) (Charrel *et al.*, 2003).

The occurrence of WNV in East Africa has been well documented, but reports of its presence in other parts of the **African continent** are scattered. To consider just a few, an early report provided serological evidence of widespread human infection with WNV in Central Africa, with up to 46% of persons positive for neutralizing antibodies (Smithburn and Jacobs, 1942). In South Africa, in 1974, an epidemic occurred over a normally arid 2500 km^2 area of Cape Province after heavy rains. Post-epidemic antibody surveys of the affected human population showed that 55% had been infected by WNV (McIntosh *et al.*, 1976). The presence of WNF across North Africa, from Algeria to Tunisia and Egypt, was noted by Savage *et al.* (1999) and Charrel *et al.* (2003).

Shirafuji *et al.* (2008) stated that, to that date, WNV had not been detected in **East Asia**, but they showed the native jungle crow (*Corvus macrorhynchos*) to be highly susceptible to inoculation with WNV strain NY99, developing peak viraemias of $10^{6.5}$ to $10^{10.6}$ PFU ml^{-1} serum.

An overview of human infections with WNV in the Old World through serological surveys and case reports shows high-to-very high seropositivities across Central Africa and from Uganda to Egypt, but with little pathogenicity and most cases asymptomatic. In certain of those countries, the human seropositivity rates remained high over a period of years, suggesting continued transmission. In South Africa, in contrast, the large outbreak of WNF in an arid area of Cape Province in 1974, which had some pathological severity, was limited in time. Where outbreaks of WNF of pathological severity occurred in Israel, Romania, southern Russia and Tunisia, the reports of medically confirmed cases and fatalities reveal that the outbreaks were localized and relatively short-lived, but in Israel they reoccurred spasmodically.

(c) Epizootics in the Old World

Infections of wild and domesticated **horses** with WNV have occurred at intervals in the region of the Camargue National Park on the Mediterranean coast of France. In the summer of 1962, serious cases of neurological disease were seen in horses and humans. Many horses were living wild and difficult to observe, but, among 50 'domestic' horses with neurological symptoms, 25–30% died (Panthier, 1968; Murgue *et al.*, 2001a,b). During 1964, an infective agent was isolated from a batch of *Culex modestus* and from two entomologists working in the field; it was identified as WNV (Hannoun *et al.*, 1964). Between 6 September and 30 November 2000, a serious outbreak among equines occurred within the Camargue in an area of marshes with colonies of migratory and resident birds and large mosquito populations. From among 129 horses and two donkeys clinically suspected of infection with WNV, 59 horses and one donkey became confirmed cases, of which 20 died. There were no human cases (Murgue *et al.*, 2001b).

Most findings of virulent infections with WNV in **domesticated geese** have been in Israel. In November 1997, an outbreak of a neuroparalytic disease caused by WNV was diagnosed in flocks of young geese (Malkinson *et al.*, 1998); an isolate collected at that time was later named Goose97 [AF380663] (Banet-Noach *et al.*, 2003). In September 1998, outbreaks of WNV affecting young geese were recorded on farms throughout Israel where the geese were reared in open farmyards, with death rates reaching 40% in some flocks. A unique feature of the Israeli isolate was its marked pathogenicity for flocks of young geese between 3 and 12 weeks of age; almost no manifest susceptibility of geese to WNV had been reported previously from Israel or elsewhere. In Israel, goose flocks were again infected in 1999 and 2000 (Malkinson *et al.*, 2001, 2002). During the 1996 epidemic of human WNF in Romania, high infection rates were found in ducks, geese, chickens, peahens and turkeys; in contrast, infection was found in birds of only one of 12 passeriform species tested (Savage *et al.*, 1999).

(d) Old World genotypes of WNV

Historical records of the first identified occurrences of West Nile virus in different countries indicate a strong likelihood that WNV arose in sub-Saharan Africa, with the development of lineages 1 and 2. From there it disseminated, principally as lineage 1 strains, to other regions, possibly first to the Sudan, Egypt and the eastern Mediterranean region. During outbreaks of West Nile fever in 1951, strains of WNV were isolated from human cases in Egypt (Egypt 101) and Israel (Maayan Zvi) (Table 45.30). Phylogenetic analyses of WNV isolates from different countries are reported here to indicate their relationships, but in knowledge of the warning that a failure to produce a sufficient number of complete sequences of Old World strains of WNV makes it difficult to clearly elucidate the evolutionary relationships of many strains (Charrel *et al.*, 2003).

An investigation by Savage *et al.* (1999) associated strains of WNV from sub-Saharan Africa with strains from distant countries. Phylogenetic analyses of 23 WNV strains were undertaken using the nucleotide and deduced amino acid sequences of small portions of the E glycoprotein gene. Bootstrap analysis of the nucleotide alignments showed 95% support for a lineage composed of strains from West, Central and East Africa, strains from northern Africa including Egypt and Algeria, a strain from Israel, and strains from France and Romania. The nucleotide sequence of an isolate from Romania (RO97-50) was virtually identical with isolates from Senegal (ArD93548) and Kenya (KN3829), while a close identity of amino-acid sequences was demonstrated between another isolate from Romania (RO96-1030) and isolates from Senegal (AnD27875), the Central African Republic (HB6343), and Algeria (ArDjanet), all four strains possessing a potential glycosylation site, Asn-Tyr-Ser, at position 154–156. The investigators considered these findings to be compatible with the introduction of WNV into Romania by birds migrating from sub-Saharan Africa to North Africa and southern Europe (and presumably to central Europe also).

Being at an intermediate location on migratory routes between Africa and Eurasia, Israel may be prone to invasion by different strains of WNV, and it may also be the source of strains that are carried to other countries, a possibility that has triggered a number of investigations. In one, the phylogenetic relationships were examined between 24 isolates of WNV collected from birds and horses in Israel from 1997 to 2001, and seven isolates from Volgograd, Romania, Italy, Egypt, Kenya and New York. The hosts were domesticated geese (15), a stork (1), a gull (1), a rosella (1), pigeons (2), equines (5), humans (4), mosquitoes (2). All isolates were of lineage 1. In a phylogenetic tree based on partial *E* gene nucleotide sequences, the isolates grouped in two clusters, I and II, with a percentage nucleotide difference between them of c. 8%. Each cluster consisted of distinct clades and sub-clades (Figure 45.32). With a single exception (NY99eq), all 19 cluster-1 isolates were from Israel. Of the 12 cluster-2 isolates, six were from Israel and six from five other widely separated Old World countries – Russia, Romania, Italy, Egypt and Kenya. A phylogenetic tree developed from the deduced amino acids yielded very similar placements of the 31 isolates, which also grouped into two clusters and largely similar clades, but the Eg1 and Romania96 isolates fell more evidently within cluster 2. Phenotypes of both clusters circulated in Israel during 1998 to 2001, but it was strains of cluster 2 that were collected in Romania, Italy and Kenya (Banet-Noach *et al.*, 2003).

An outbreak of neuroparalytic disease in flocks of young domesticated geese in Israel in November 1997 was shown to be caused by WNV. The WNV isolate named goose97 was collected in that year, and further closely related isolates (e.g. goose98) appeared each year until 2001. Genetically distinct isolates (e.g. GooseKY98 and GooseMaV00) were isolated between 1998 and 2000 (Banet-Noach *et al.*, 2003). The 1998 outbreak in geese started about a month after the arrival of a flock of infected white storks at Eilat during August (Section 45.7.8.d). The *E* gene sequences of the goose98 and goose99 isolates and the stork-1998 isolate from Eilat showed only three nucleotide differences between them, while their *E* gene amino acid sequences were identical (Malkinson *et al.*, 2002).

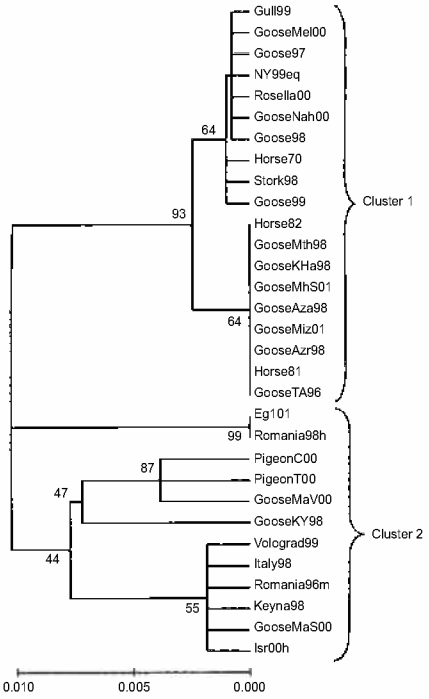


Figure 45.32 Phylogenetic tree of isolates of West Nile virus from Kenya, Egypt, Israel, Italy, Romania and Russia. (From Banet-Noach *et al.*, 2003; derived in part from Lanciotti *et al.*, 1999.) The tree was generated by neighbour-joining analysis of 1278 bp of the *E* gene by the Kimura-2 parameters method. The number at each node indicates the percentage of 1000 bootstrap replicates; the scale represents the number of genetic substitutions per genomic site. The names of isolates are those provided by the investigators, and in some cases differ from those used by other authors, but may be identified more precisely by their GenBank Accession numbers. **Isolates from Israel.** *Geese*: Goose 97 [AF380663]; Goose98 [AY033388]; Goose99 [AY033391]; GooseAza98 [AY052413]; GooseAzr98 [AY052410]; GooseKHa98 [AY052409]; GooseKY98 [AY052412]; GooseMaS00 [AF380667]; MaV00 [AF380666]; GooseMe100 [AF380664]; GooseMhS01 [AY184820]; GooseMiz01 [AY184821]; GooseMth98 [AY052411]; GoosNah00 [AF380665]. GooseTA98 [AY052408] *Other birds*: Stork98 [AY033389]; Gull99 [AY033390]; Rosella00 [AF380668]; PigeonC00 [AF389671]; PigeonT00 [AF380670]. *Equines*: Horse70 [AF380669]; Horse81 [AY052407]; Horse82 [AY052406]. *Humans*: Isr00h [AF394217]. **From elsewhere.** *Kenya*: Kenya98 (from *Culex*) [AF146082]. *Egypt*: Eg101 (from human) [AF260968]. *Italy*: Italy98 (from horse) [AF404757]. *Romania*: Romania 96m (from *Culex*) [AF130362]; Romania96h (from human) [AF130363]. *Russia*: Volgograd99 (from human) [AF317203]. *United States*: NT99eq [AF260967].

After the WNV epidemic in Israel in 2000, isolates were obtained from deep-frozen sera kept from four human cases. Sequence and phylogenetic analysis of nucleotides of the *PrM* and *M* genes and part of the *E* gene showed that infections had been caused by two different strains of WNV. A search of the GenBank database, using equivalent fragments to those of the Israeli outbreak isolates, revealed relationships of the two strains to isolates from other countries. (i) Two isolates from north and north-central Israel, named WN0304 and WN0233 (Table 45.30), showed 98% homology with the isolate RO97-50mos from *Culex pipiens* in Romania and the isolate Russia-1999 from a human host in Volgograd, indicating associations with central and eastern Europe. (ii) Two isolates from central Israel named WN0043 and WN0247 showed 99.7% homology with five isolates, all collected in 1999 before the epidemic: from Israel, gull99; from New York, NY99-flamingo, NY 1999-human, and NY99-equine, and from Connecticut, Conn-1999 from *Culex pipiens* (Bin *et al.*, 2001; Hindiyeh *et al.*, 2001).

45.7.4 West Nile virus in the New World

(a) Arrival and dissemination

The first appearance of WNV infections in the western hemisphere occurred in and around New York City during the summer and early autumn of 1999. Avian deaths from WNV began in July (Epstein and Defilippo, 2001), and the earliest confirmed human case of WNF presented on 4 August. The dates of onset of confirmed human cases peaked in late August, and there were no recognized cases with a date of onset after 22 September (CDC Reports*). The first eight human cases lived within a 41.6 km^2 area in the northern Queens borough of New York City (Nash *et al.*, 2001). The distribution of human cases spread within New York State during 1999, and by the end of the period of transmission 62 cases of WNF had been notified, most of them serious, with seven fatalities. Avian infections with WNV occurred more extensively, and during 1999 were reported from four States – New York, New Jersey, Connecticut and Maryland (Fine *et al*, 1999a,b; Nash *et al.*, 2001; Anon., 2010). Many human infections with WNV were asymptomatic, and it was estimated that 2.6% of the residents of Queens might have been infected (Garmendia *et al.*, 2001). Mosquitoes were found in all areas of New York City where human cases occurred except Manhattan. Mosquito larval habitats were found in the yards and neighbourhoods of the first eight cases who lived in Queens. Most of the mosquito pools from which WNV was isolated included extracts of *Cx. pipiens* and *Culex restuans* (Nash *et al.*, 2001).

In 2000, among a total of 21 human cases attributed to WNV, 19 suffered severe neurological illness and there were two fatalities (Table 45.32, A). The epicentre of WNF was again New York City and surrounding counties, but human cases were reported from three states – New York, New Jersey and Connecticut. Epizootic activity in birds and/or mosquitoes preceded the onset of human illness in all of these areas, but the dissemination of WNV had been wider, with infected mosquitoes recorded in five states and infected birds in 12 states (Novello *et al.*, 2000; Hayes *et al.*, 2005a; Anon., 2010). The outbreak in New York heralded a spread of WNV towards the north, west and south.

In 2001, human infections with WNV were reported from ten states, with a total of 66 cases. In 2002, human infections were reported from 39 states and the District of Colombia, with a 63-fold increase in cases to 4156 (Table 45.32, B). For 2004, the CDC reported human infections in 40 states, but, having assembled data from mosquitoes, birds and mammals, Davis *et al.* (2005) stated that by 2004 WNV was present in all 48 contiguous states of the USA (as also in seven Canadian provinces, Mexico, the Caribbean islands and Colombia). The

*Centers for Disease Control and Prevention. Records of human disease incidence and distribution are published by the CDC in issues of their *Morbidity and Mortality Weekly Report*. The published data could be altered later, so the data noted here, in the text and in Table 45.32, have been corrected where necessary by reference to the archive for the period 1 January 1999 to 31 December 2009, as reported through to 30 April 2010, to ArboNET, and published by CDC. That source is cited in the text as Anon. (2010).

Table 45.32 Total human cases of West Nile fever in the USA, including both mild and severe, reported to the CDC Surveillance Program from 1 January 1999 to 31 December 2009. (Available at: http:www.cdc.gov/ncidod/dvbid/westnile/surv&control_archive.htm)

A. Categories of infection by year

Year	*Neuroinvasive cases* * (n)	*West Nile fever cases* † (n)	*Clinical/ Unspecified* ‡ (n)	*Total cases* (n)	*Fatalities* (n)	(%)
1999	59	3	0	62	7	11.3
2000	19	2	0	21	2	9.5
2001	64	2	0	66	10	1.5
2002	2946	1160	50	4156	284	6.8
2003	2866	6830	166	9862	264	2.7
2004	1142	1269	128	2539	100	2.5
2005	1294	1607	99	3000	119	3.9
2006	1459	2616	194	4269	177	4.1
2007	1217	2350	63	3630	124	3.4
2008	687	624	45	1358	44	3.2
2009	373	322	25	720	32	4.4

B. Number of states recording human WNV infections by year, and totals of cases by year

	1999	*2000*	*2001*	*2002*	*2003*	*2004*	*2005*	*2006*	*2007*	*2008*	*2009*
No. of states	1	3	10	39	45	40	43	43	43	45	38
Total cases	62	21	66	4156	9862	2539	3000	4269	3630	1356	730

For Table A: *, Cases of West Nile encephalitis or West Nile meningitis; †, West Nile fever cases refers to less severe cases that showed no evidence of neuroinvasion; ‡, Either clinical cases with manifestations other than WNF or cases for which insufficient clinical information was provided.

distribution of human cases remained extensive and the numbers high (43 in 2005–2007, and 45 in 2008) until 2009, when the number of states recording human infections fell to 38, and the total numbers of cases fell by 47%. Evidence suggests that the numbers of clinically confirmed cases of infection in humans are a minute percentage of the many asymptomatic infections.

A comparable appearance and spread of WNV infections over time was recorded in horses. From August to November 1999, a single outbreak occurred in an epicentre of 4 km radius on Long Island, relatively distant from New York City, when 31 horses showed a range of neurological symptoms, including staggering, incoordination, muscle fasciculations and recumbence. The brain of one of the horses showed encephalitic lesions and was infected with WNV. Of 29 serologically tested horses, 20 were positive for anti-WNV antibodies, out of which four died (Garmendia *et al.*, 2001; Trock *et al.*, 2001). During 2000, veterinary surveillance identified WNV infections in 58 horses exhibiting severe neurological disease, spread over seven north-eastern states but mostly in New Jersey and New York (Novello *et al.*, 2000). In 2001, of the 733 equine cases reported more than two-thirds were in Florida; whereas in 2002 the 9144 equine cases reported were more evenly distributed between Illinois, Texas, Minnesota, Indiana, Kansas and South Dakota (Ward, 2005). Following the licensing of an equine WNV vaccine in 2003, immunization eventually greatly decreased the number of cases. Humans and horses are tangential hosts of WNV, and at most their effects upon the transmission cycle of the virus would be to reduce slightly the number of vectors available to

feed on its amplifying hosts, so possibly dampening any epidemic (Figure 45.33).

During the 1999 outbreak in New York, epizootics of WNV occurred in populations of wild birds in New York State, Connecticut and New Jersey. The strain of WNV was highly virulent to some native bird species, notably American crows (*Corvus brachyrhynchos*), the dead bodies of which were conspicuous. At the Bronx Zoo and Queens Wildlife Center, deaths from WNV infection occurred among 14 species of birds, both native and exotic (Steele *et al.*, 2000). During 2000, surveillance of birds showed extensive epizootic activity, centred in New York City metropolitan area but extending throughout much of the eastern seaboard, from southern New Hampshire to North Carolina. In 2001, epizootic activity in birds was recorded from twelve states and the District of Colombia (Lanciotti *et al.*, 1999; Nash *et al.*, 2001). During 2002, 14,122 dead birds submitted from 42 states were WNV infected, of which 7719 were American crows, 4948 were blue jays (*Cyanocitta cristata*) and 1455 were birds of 92 other species. Of the American crows submitted, 77% were WNV positive, compared with 40% of birds of other species (Chow *et al.*, 2002).

(b) Comparison between the epidemiologies of WNF in the USA and regions of the Old World

The records of the numbers and distribution of clinically evident human cases of WNF in the USA during the period 1999 to 2009, summarized in Table 45.32, provide a realistic indication of the dissemination and persistence of WNV in that country over time, and can be used to compare the epidemiology of West Nile fever in the USA with that in regions of the Old World. Unfortunately for any broader understanding of the disease, those figures are no guide to the epizootiology of WNV, which requires information on the numbers and geographical distributions of infections in the amplifying hosts and mosquito vectors of the virus.

The spread of WNF to 39 continental states of the USA during 2002 was associated with a massive increase in the number of human cases, and for the following 6 years the annual totals of human cases remained high and their distribution extensive.

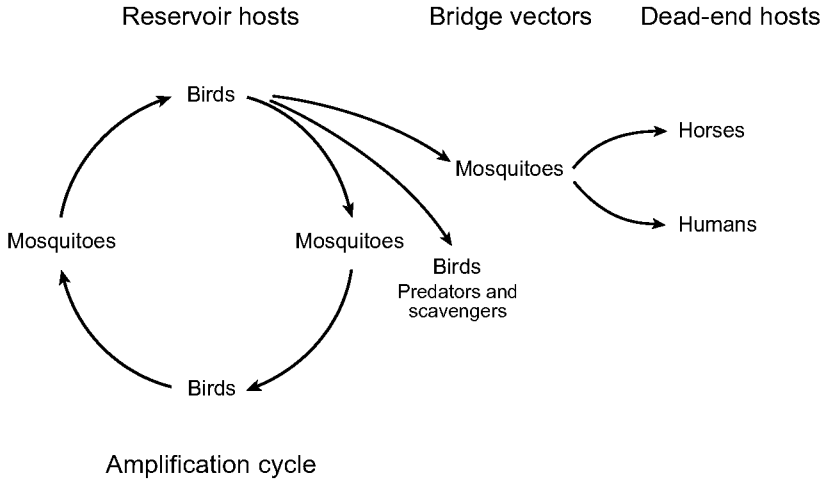


Figure 45.33 Transmission cycles of West Nile virus in North America, by which WNV is transmitted between mosquitoes (most importantly species of *Culex* subgenus *Culex*) and certain species of wild birds. Bridge vectors transmit the virus to humans and horses, which are dead-end hosts. Probably there is additional transmission to predatory or scavenging birds that feed on WNV-infected birds, and to birds that ingest bird faeces containing WNV. The arrows denote paths of virus transfer.

That was in apparent contrast to the spasmodic outbreaks of WNF in Israel, Central Europe and North Africa during the 1950s to 1990s. Taken overall, the prevalence of infection in the USA was not particularly high; the greatest overall prevalence occurred in 2003, when 9862 cases were reported from 45 states, a mean of 219 cases per state; the next highest prevalence had occurred the year before, in 2002, with a mean of 106 cases per state.

What appears exceptional was the continuing occurrence of WNF in 39 or more states for a period of 7 years. At present, the reasons for this pattern of prevalence over time can only be a matter of conjecture. It could be that the ecological situation across North America was sufficiently favourable to maintain the transmission cycle of WNV, e.g. through the wide distribution of avian amplifying hosts with high host competence under natural conditions (Section 45.7.7.c), and the near-universal presence of mosquitoes with high vectorial capacity. The records of human WNF across the USA are based on numbers of clinically identified cases (Table 45.32), in addition to which there would have been a very much higher number of unrecorded, asymptomatic infections. Across Central Africa and from Uganda to Egypt, where relatively few clinically confirmed cases of human WNF were recorded, serological screening revealed the continuing presence of asymptomatic infections in humans over periods of years. That the New World strains of WNV were more virulent in humans than most Old World strains had been might account, to some extent at least, for the difference between the New and Old World epidemiologies.

(c) North American genotypes of WNV

The results of phylogenetic analysis of complete or partial genomes of a number of US and Israeli strains strongly supported the hypothesis that North American strains of WNV were derived from an Israeli strain introduced into the USA. Analysis of nucleotide sequences of the *pr*M and *E* genes of isolates from New York and Israel revealed a close relationship. A similarity of 99.8% or more existed between nine isolates collected in and around New York in 1999 and an isolate collected from the brain of a dead goose in Israel in 1998 that was named WN-Israel 1998 [AF205882]. The New York isolates included one from a Chilean flamingo that died in Bronx Zoo, NY99-flamingo [AF196835], and others from the American crow, *Culex pipiens*, and equine and human hosts. The marked pathogenicity for birds exhibited by the Israeli strain was shared by the New York strain (Lanciotti *et al.*, 1999). The close relationship between Israeli and New York isolates was confirmed when Lanciotti *et al.* (2002) reported that eight isolates obtained in the USA during 1999 and 2000 shared ≥99.7% nucleotide and amino acid identities with the 'Israel 1998-stork' isolate [AY033389] (Table 45.30).

Certain Old and New World strains of WNV were not genomically remote. In a phylogenetic tree based on E-glycoprotein gene fragments of isolates from 17 countries, three lineages – WN-Israel 1998 [AF205882], NY99-flamingo [AF196835] and 'Tunisia 1997' – formed a minor clade. The Tunisian isolate was from a WNF patient in an outbreak on the coast of that country, in which eight of 173 patients died (Murgue *et al.*, 2001a). Later, the Tunisian isolate was analysed and named PaH001 [AY268133]. In a phylogenetic tree generated from complete nucleotide sequences, a cluster of viruses comprising one from Israel and a number from the USA was rooted by PaH001 (Charrel *et al.*, 2003).

Together, these findings indicate that the first outbreak of West Nile fever in North America resulted from a single introduction of WNV. Other biological evidence was consistent with the amplification of WNV in a transmission cycle involving native mosquitoes and birds.

Isolates of WNV collected in the New York area during 1999 and 2000 displayed a high degree of sequence conservation, with a nucleotide identity of ≥99.8% and an amino acid identity of ≥99.9% (Lanciotti *et al.* (2002), but subsequently new genotypes arose. Ebel *et al.* (2004) reported on genetic and phenotypic variation of WNV in the New York area during 2000–2003, as revealed from

analyses of the *E* gene coding regions of 67 isolates and of fragments of the *NS5* genes of 39 isolates. Phylogenetic analysis grouped the isolates into two clades: one comprised isolates collected during 1999–2003 and was designated NY99; the other comprised isolates collected solely during 2002 and 2003 and was designated WN02. In fact, WN02 had been isolated earlier than 2002. A phylogenetic tree based on analysis of a 2004-nucleotide sequence from 22 isolates collected in four widely separated states showed all to be of WN02. Of the total, two isolates had been collected during 2001 and 20 during 2002. The two 2001 isolates, which had been collected in Alabama from *Cx. quinquefasciatus* and crows, were named Alabama-1 and Alabama-2, respectively (Davis *et al.*, 2003). Those two isolates were regarded as an 'intermediate sister clade' of WN02 by Davis *et al.* (2005). However, from calculations of mean rates of evolutionary change for NY99 and WN02, Snapinn *et al.* (2007) considered it probable that WN02 had arisen before it was first detected in 2001, but did not become epidemiologically significant until 2002.

Insights into developments during the period 2001 to 2004 were provided by analyses of partial nucleotide sequences of the *prM* and *E* genes from 108 isolates collected in the USA and Canada. A phylogram based on the findings grouped the isolates into three clades. (i) A small 'Eastern US clade' comprising isolates collected from New York and the eastern US states from 1999 and mostly before 2003. This included the isolates NY99-equine, NY1999-human and Conn-1999 (Table 45.30). (ii) A smaller 'Southeast-coastal Texas clade', closely related to the Eastern US clade, but which by 2005 was possibly extinct or displaced, and included Bird 9483 (Table 45.30). (iii) A very large clade named the 'North America 2002–2004 clade' (≡ the WN02 clade of Ebel *et al.*, 2004), comprising isolates from all over North America that were collected from 2002 to 2004, and which included Mosq. V4369 and AZ 2004 (Table 45.30). This included many sub-clades, each comprising isolates most of which had been collected in the same state and during the same year (Davis *et al.*, 2005).

A later analysis of 44 WNV isolates obtained in the metropolitan area of Houston, Texas, during the years 2002–2006, showed that all 44 isolates contained the three nucleotide mutations in the *prM* and *E* genes that differentiated WN02 from other genotypes. Most isolates from 2002 had the shortest branch lengths from the node separating the NY99 and WN02 clades, suggesting that they represented early stages in the emergence of the dominant genotype. The isolates from 2003 to 2005 had, on average, longer branch lengths than those of 2002, but only a few of those isolates had more than three nucleotide mutations from the dominant clade-defining node, which suggested that WNV had reached a relative genetic stasis at the local level (Davis *et al.*, 2007).

By 2004, WN02 had disseminated through all continental states of the USA and beyond, becoming epizootically dominant; between 2001 and 2004 it completely displaced the NY99 genotype, after which time the latter was no longer found. Experimental findings revealed a significant phenotypic difference between the two clades. With *Cx. pipiens* as host, and kept at 27 °C, the extrinsic incubation period of WN02 was 2–4 days shorter than that of NY99; consequently, WN02-infected hosts transmitted the virus about 2 days before hosts infected with the competing NY99 form (Ebel *et al.*, 2004). Similar experiments showed that, with *Cx. tarsalis* as host, WN02 was transmitted 4 days sooner than NY99 (Moudy *et al.*, 2007).

To compare the growth rates of different populations of WNV in North America, a coalescent analysis was undertaken with comparison of the complete *E* coding region sequences of isolates of NY99 and WN02 collected between 1999 and 2005. Estimates of the rates of nucleotide substitution showed similar mean rates of evolutionary change for NY99 and WN02 at *c.* 3×10^{-4} nucleotide substitutions per year. At those rates, the mean ages of the 'genetic diversities', i.e. of the most recent common ancestors, were 8 years for NY99 and 6 years for WN02, and were compatible with epidemiological records. Estimates of fit of the NY99 and WN02 genotypes to four

demographic models of population growth were determined, the models being constant population size, exponential growth, logistic growth and expansion growth. For NY99, a model of exponential growth gave the best fit, whereas the demographic history of WN02 fitted a model of logistic growth, in which an initial phase of rapid population growth is followed by a slowdown in growth rate. The rapid-growth phase of WN02 corresponded to a mean growth rate of six new infections per host per year, or an epidemic doubling time of about a month. In contrast, the equivalent values for the logistic growth of NY99 were only two infections per host per year and a doubling time of about 5 months, which could account for the rapid displacement of NY99 by WN02. Following the disappearance of the NY99 genotype, there was a marked decrease in the growth rate of WN02, which was taken to signify that WNV had reached its peak prevalence in North America (Snapinn *et al.*, 2007).

(d) Effects of ambient temperature

Recorded outbreaks of human West Nile fever in the northern hemisphere mostly occurred within a 3 month period that fell between mid-summer and late autumn, as the dates of the following outbreaks show: Israel 1951–1957, 1980, 2000 – late July to early October; Romania 1996 – mid-July to mid-October; Tunisia 1997 – September to November; southern Russia 1999 – July to September; New York 1999 – early August to late September. In certain cases, the outbreaks were reported to have followed a period of high temperature. For example, during May and June 1996 the average daily temperature in Bucharest was higher than in any of the years 1991 to 1995, preceding an outbreak that started there in July (Savage *et al.*, 1999). For 3 weeks in July 1999, temperatures in New York were 4.6°C above the 30-year average; avian deaths from WNV were first observed during July and human cases first occurred in August (Epstein and Defilippo, 2001).

Temperature affected the dispersal and amplification of WNV during the years 2002 to 2004, as it disseminated through central and western North America. Maps depicting deviations of summer temperature from a 30-year mean revealed that WNV always dispersed into new areas during years with above-normal temperatures, and that amplification during the following year occurred during summers with above or normal temperatures. Subsequent cool summers were associated with decreased or delayed virus activity. At southern latitudes, in Louisiana and Arizona, outbreaks were not associated with above-normal temperatures, probably because the average summer temperatures were sufficient to allow effective transmission (Reisen *et al.*, 2006a).

Two attempts were made to explain these temperature effects. Reisen *et al.* (2006a) used a degree-day model to analyse experimental data, degree days being the number of days since ingestion of an infectious blood meal multiplied by the ambient temperature in °C above a minimum temperature threshold below which no transmission is assumed to occur. WNV of the NY99 strain was ingested by females of *Cx. tarsalis* feeding on viraemic passerine birds. The mosquitoes were kept at fixed temperatures between 14°C and 30°C, and over that range a correlation was found between temperature and the extrinsic incubation period (EIP). From regression of the EIP rate (the inverse of the EIP) as a function of temperature between 14°C and 30°C, the minimum temperature threshold was estimated to be 14.3°C, and the relevant number of degree days to be 109. The model indicated that transmission of the NY99 strain could not commence until, with increased temperature, the EIP had shortened to less than the duration of two gonotrophic cycles; effectively, therefore, not until the female took a third blood meal.

Kilpatrick *et al.* (2008) considered the traditional degree-day model to be inappropriate, arguing that the rates of the chemical and kinetic processes involved in viral infection, replication and transmission would increase exponentially with temperature, so that the relationship between number of degree days and extent of transmission should accelerate with increase in temperature. This supposition was tested experimentally with the

WNV genotypes NY99 and WN02 with colonized *Cx. pipiens* as vector. A regression model was built where T = ambient temperature in °C, t = time as days since feeding, and tT = degree days. It was anticipated that transmission would increase exponentially with temperature as e^T, where e is the base of the natural logarithm. The term tT was raised to the power of n (tT^n) (n representing any selected value), revealing the effect of increases in n. For studies of genotype interaction, estimates of degree days were used with T also raised to the nth power. The experimental data showed that low-level transmission occurs at 14–15°C, but only after periods that might exceed lifespan in the field. Transmission of both strains of WNV accelerated sharply with increasing temperature, such that small increases in temperature had relatively large effects on transmission. The results showed that traditional degree-day models might not accurately describe the impact on transmission, but that with the data used by Kilpatrick *et al.* (2008) a degree-day term with temperature raised to the fourth power, tT^4, was most accurate in explaining transmission. The results were taken to show that both viral evolution and temperature help shape the distribution and intensity of transmission of WNV. The advantage of the WN02 over the NY99 genotype increased with the product of time and temperature, so warmer temperatures would have facilitated the invasion of the WN02 genotype.

45.7.5 Mathematical modelling

(a) Basic reproduction rate

In developing a mathematical model of the epidemiology of malaria, Ross (1911) and Macdonald (1952a,b,c, 1953, 1955, 1957) introduced the concept of the 'basic reproduction rate' (z_0, later R_0), being the average number of secondary infections distributed in a human community as a direct result of the introduction of a single, primary, non-immune case. Thus,

$$R_0 = \frac{ma^2 bp^n}{r(-\log_e p)} \qquad (45.6)$$

where, for transmission of malaria parasites, m is the relative density of vectors per person, a is the average number of persons bitten by a named vector in one day, b is vector infectivity, p is the daily survival rate of the vector, r is the human recovery rate and n is the extrinsic incubation period. The equation can be broken down into epidemiological terms: ma combines the relative density of vectors (m) with their biting rate (a) which enters twice in each cycle, hence a^2. Life expectancy is $1/-\ln p$; and the primary case would be infective for $1/r$ days.

Later, this was recognized as a key concept in epidemiology, and it has been used in epidemiological investigations of a variety of infectious diseases. Usually, the basic reproduction rate, R_0, has been taken to indicate 'the number of individuals infected by a single infected individual during its entire infective period in a population that is entirely susceptible'. When $R_0 < 1$, each infected individual produces, on average, less than one new infected individual, so the infection will be cleared from the population. When $R_0 > 1$, the pathogen is able to invade the susceptible population. The magnitude of R_0 has been used to gauge the risk of epidemics arising from emerging infectious diseases (Heffernan *et al.*, 2005). How far quantitative values determined from use of the basic reproduction rate are accurate may be questioned, but the structure of the equation has provided an invaluable guide to understanding interactions between infectious agent, vector and host.

Restriction of Eqn 45.6 to its entomological terms results in an expression of vectorial capacity, C, such that

$$C = \frac{ma^2 p^n}{-\log_e p} \qquad (45.7)$$

which is the average number of potentially infective bites that will ultimately be delivered by all the vectors that feed upon a single infective host in one day.

The development of mathematical models of disease transmission requires the coupling of mathematical insight with the provision of suitable

biological data and, critically, an informed interpretation of the data. The use of laboratory-derived experimental data at the developmental stage of a model, or when running a model, is likely to reduce the validity of the outcome, and for only a few vector species have life expectancy measurements been obtained for wild populations that were exposed to a range of natural hazards. Further, in models of WNV transmission, R_0 depends on features associated with a mosquito–bird transmission cycle with multiple amplifying hosts, a more complex situation than human malaria with its single amplifying host; consequently, there is greater reliance on laboratory data.

(b) Population densities and WNV transmission

Noting that WNV in North America exhibits a complex seasonal ecology which is not readily analysed with standard epidemiological methods, Wonham *et al.* (2004) derived a system of differential equations to develop a single-season SIR (susceptible–infective–recovered) model of cross-infection of WNV between its avian hosts and mosquito vectors. The model is dynamic in the sense that individual hosts are born susceptible, then become infected and infective, and finally recover. An epizootic stops when the number of susceptibles declines. By determining the magnitude of R_0 for vector–host–vector or host–vector–host phases of transmission, or alternatively determining it as the square root – which indicated the geometric mean for a combination of average individuals of the vector and host species, the relative effects of different control procedures on the magnitude of R_0 and therefore on WNV transmission can be determined. It appeared that mosquito control should decrease the chance of an outbreak of WNF, whereas control of the population density of an avian host should increase it. (As expressed by Bowman *et al.* (2005), a mosquito control operation that results in $R_0 < 1$ can lead to the eradication of WNV from the transmission cycle, whereas a reduction in the size of the amplifying host population results in $R_0 > 1$ and leads to persistence of WNV in the cycle.) Extending the model to a seasonally variable mosquito population enabled Wonham *et al.* (2004) to outline a so-called multi-year model. The non-linear relationship between the adult mosquito infective lifespan and the 'critical equilibrium mosquito level' showed that a small increase in mosquito mortality could lead to a disproportionately large increase in the outbreak threshold. In contrast, a reduction in crow population density should enhance transmission of the infective agent, because R_0 scaled positively with the mosquito:bird ratio.

(c) Virulence and WNV transmission

Foppa and Spielman (2007) made use of the basic reproduction rate in modelling the effects of the virulence of WNV on the mortality of its avian hosts, rewriting Eqn 45.6 as

$$R_0 = \frac{ma^2 d\, p^n b_m b_h}{-\ln p} \tag{45.8}$$

where m is the mosquito:host ratio, a the biting rate of a female mosquito, d the duration of infectiousness in human hosts, p the daily survival rate of the mosquitoes, and n the duration of the extrinsic incubation period in days. The variables b_m and b_h quantify transmission from an infective mosquito to a susceptible human host and from an infective person to a mosquito. Quantitative values for the variables were adopted from published sources, some of the data being from laboratory experiments.

To explore the relationship between virulence and the dynamic aspects of transmission, epizootics were simulated under conditions that ranged from extremely virulent (no bird survival) to avirulent (100% survival), with intermediate scenarios of 10%, 25%, 50%, 75% and 90% survival. The magnitude of R_0 increased with increasing virulence, so the mortality of avian amplifying hosts rose owing to an increasing mosquito:host ratio. Higher virulence was consistently associated with an increase in the number of infective mosquitoes at the end of the simulated epizootic. Invariably, all

birds became infected and either died or, if surviving, became immune, so no susceptible birds remained. That was consistent with the finding of Wonham *et al.* (2004) (in Section 45.7.5.b above) that R_0 increases with mortality of the amplifying host due to the increasing mosquito:host ratio. They postulated that reducing crow densities should enhance WNV transmission because R_0 would scale positively with the mosquito:bird ratio.

In the field, host mortality will translate into a higher R_0 only if the increase in the mosquito:host ratio results in an increase in the average number of blood meals taken per bird. To examine the impact of 'vectorially incompetent' host species on transmission dynamics, the effect of extreme virulence on R_0 in the presence of various population densities of alternative hosts was explored. The simulated presence of alternative hosts, even when scarce, reduced the transmission-boosting effect of host mortality. However, the dynamics of transmission between birds were only moderately affected by the level of virulence owing to the high starting value of R_0, which effectively guaranteed universal infection. This finding was inconsistent with the suggestion that the death of 'vectorially competent' hosts had contributed to the rapid spread of WNV over North America. Foppa and Spielman (2007) concluded that the assumptions upon which that argument was built need to be empirically examined.

(d) Transmission assumptions

Analysis of seven dynamic models of mosquito-borne arbovirus transmission, five of them modelling WNV transmission, showed that, generally, differences in assumptions about biological features resulted in differences in predictions of disease outbreak. In determination of the basic reproduction rate, the definition of any one of the terms that constitute its mathematical expression (Eqn 45.6) can quantitatively alter the rate. Thus, R_0 could be influenced by different assumptions concerning epidemiological features such as characteristics of the amplifying host or the life history of the vector. In a comparison of different mathematical models, *Culex pipiens* was used preferentially as the vector, while measurements of mortality and recovery rates were obtained for six common North American bird species as amplifying hosts. Biological features that affected R_0 included the use of single or multiple amplifying host species, and the life history characteristics of the vectors. Changes of transmission functions in the core model (Eqn 45.6) led to numerical estimates of R_0 that ranged over an order of magnitude or more. Wonham *et al.* (2006) argued that such conflicting predictions could be reconciled by appreciating that each disease-transmission term could realistically be applied only to a certain range of vector and (so-called reservoir) amplifying-host population densities.

(e) Caution

Interactions between coexisting natural populations of a host and a parasite may generate selective forces that lead to genetic modifications, with phenotypic changes to the virulence of the parasites and the severity of host pathology. In other words, there can be a trade-off between the variables of virulence and pathology (Section 41.5.1). Such a trade-off between aggressiveness of the parasites and the pathological condition of the hosts has been best documented from interactions between natural populations of myxoma virus and European rabbits in Australia (Section 43.4.3). Knowledge that genetic and phenotypic changes occur in interacting populations of hosts and pathogens points to the need for caution against accepting the findings from epidemiological models as more than a guide. Further, when a model is fed with a combination of data from natural populations and laboratory populations, the latter having only a remote association with the natural agent or the vector – as is often the case, the risk of coming to erroneous conclusions is almost certain.

45.7.6 Remote sensing from satellites

Satellite-borne sensors that record the spectral characteristics of the earth's surface, particularly its

vegetation, and that yield a vegetation index (VI) have been used to forecast the sites of future outbreaks of disease, including malaria and certain arboviral diseases. The normalized difference vegetation index (NDVI) has been used in epidemiological investigations of Rift Valley fever (Section 45.6.3.c). A later development, the Enhanced Vegetation Index (EVI), was used in an investigation of the distribution of West Nile fever outbreaks in human populations. It is defined as

$$EVI = G \times \frac{\rho_{nir} - \rho_{red}}{\rho_{nir} + (C_1 \times \rho_{red} - C_2 \times \rho_{blue}) + L} \quad (45.9)$$

where ρ_{blue}, ρ_{red} and ρ_{nir} represent reflectance in the blue, red and near-infrared bands, respectively, and C_1 and C_2 are coefficients used to correct aerosol scattering in the red band by use of the blue band. In general, G = 2.5, and L = 1. The factor L adjusts for reflectance changes from bare soil surfaces, but it increases sensitivity to topography (surface features), especially change of surface orientation from horizontal to inclined. Variations in radiance produced in that way should be removed from reflectance data before the EVI is calculated (Matsushita *et al.*, 2007).

For the states of Illinois, Indiana and Ohio, and the periods July to September 2002 and 2003, remotely sensed data for NDVI and EVI from the NASA–MODIS/TERRA data set were coupled with information from digital maps from the US Geological Survey that summarized aspects of topography, and together they were scrutinized to search for ecological factors that might be consistently associated with the transmission of WNV to humans. The NDVI values were closely similar to the EVI values, but the latter were chosen for the final analyses. Virtually all tests accurately predicted distributions of West Nile fever, suggesting that one or more elements in the WNV transmission cycle were governed by ecological factors. Areas that the remote sensing data predicted should be suitable for WNV transmission tended to have lower than average values of EVI during the summer months (Peterson *et al.*, 2008).

45.7.7 Avian hosts

Bird species known to be naturally infected with West Nile virus are listed in Table 45.33.

(a) Virulence

The concept of virulence is discussed in Section 41.5.1. In a number of zoogeographical regions, many species of native birds are susceptible to infection with WNV. In most regions of the Old World, the virulence of local strains of WNV to birds has been low, but that is not the case in North America, where the local strains of WNV are highly virulent to some birds.

That the strain of WNV that appeared in New York in 1999 was virulent to birds was soon apparent from the abundance of dead bodies of a number of species, especially the larger corvids. From mid-July 1999 through to 2005, the years in which WNV disseminated across the USA and became established, 21,496 dead birds were submitted from 45 states for examination through the ArboNET surveillance system, of which 81% were corvids and 19% other species. Of the dead birds, 8653 were tested for WNV and 5344 (24.9%) were found positive. The infected birds included 240 native species, some 27% of all native species, but in an unknown proportion of those species the death rates from WNV infection may have been very low. The so-called 'top five' WNV-positive species, all native species and all but one corvids, were the: American crow, 39%; blue jay, 17%; western scrub-jay (*Aphelocoma californica*), 16%; yellow-billed magpie (*Pica nuttalli*), 7%; and house sparrow, 3% (82% of the total infected) (Farnon, 2006).

The virulence of WNV in certain birds, and the associated elevated viraemias in several passerine species, especially within the Corvidae, have been considered important factors in the infection of *Culex* species and the transmission of WNV. Not only do corvids produce the most elevated viraemias, but the incidence of human WNF in suburban/urban habitats seems to be associated with the communal roosts of some corvids and the

Table 45.33 Bird species known to have been naturally infected with West Nile virus that are mentioned in Section 45.7.

Order Anseriformes	**Order Phoenicopteriformes**
Anatidae	**Phoenicopteridae**
Anser anser [var. *domesticus*] (goose)	*Phoenicopterus chilensis* (Chilean flamingo)
Anas platyrhynchos [var. *domesticus*] (duck)	
	Order Falconiformes
Order Charadriiformes	**Falconidae**
Laridae	*Falco tinnunculus* (common kestrel)
Larus delawarensis (ring-billed gull)	
Ichthyaetus leucophthalmus (white-eyed gull)	**Order Passeriformes**
	Corvidae
Order Ciconiiformes	*Aphelocoma californica* (western scrub-jay)
Ardeidae	*Cyanocitta cristata* (blue jay)
Ardeola grayii (Indian pond-heron)	*Corvus brachyrhynchos* (American crow)
Nycticorax caledonicus (rufous night heron*)	*Corvus corax* (common raven)
Bubulcus ibis (cattle egret)	*Corvus corvix* (hooded crow)
Ciconiidae	*Corvus ossifragus* (fish crow)
Ciconia ciconia ciconia (white stork)	*Pica hudsonia* (black-billed magpie)
	Pica nuttalli (yellow-billed magpie)
Order Columbiformes	**Fringillidae**
Columbidae	*Carpodacus mexicanus* (house finch)
Columba livia (rock dove)	**Icteridae**
Zenaida macroura (mourning dove)	*Agelaius phoeniceus* (red-winged blackbird)
Streptopelia senegalensis † (laughing dove)	*Molothrus ater* (brown-headed cowbird)
	Quiscalus quiscula (common grackle)
Order Galliformes	**Cardinalidae**
Odontophoridae	*Cardinalis cardinalis* (northern cardinal)
Callipepla californica (California quail)	**Mimidae**
Callipepla gambelii (Gambel's quail)	*Dumetella carolinensis* (grey catbird)
Phasianidae	**Passeridae**
Gallus gallus domesticus (domestic fowl)	*Passer domesticus* (house sparrow)
Meleagris galloparvo (domesticated turkey)	**Turdidae**
	Turdus migratorius (American robin)
Order Strigiformes	*Catharus ustulatus* (Swainson's thrush)
Strigidae	**Emberizidae**
Bubo virginianus (great horned owl)	*Spizella arborea* (American tree sparrow)

The scientific names accord with 'Avibase – the world bird database', at http://avibase.bsc-eoc.org/.
*, Nankeen night-heron of some authors; †,*Spilopelia senegalensis* or *Stigmatopelia senegalensis* of some authors; once known as palm dove.

sickness or death of those birds in adjacent neighbourhoods (Reisen *et al.*, 2006c). Supporting evidence of a connection was provided by an investigation at Davis, California, where 13 sites were selected for observation, seven sites had records of WNV-positive dead corvids (the so-called test sites), and six had no such records (the control sites). It was postulated that, if an infected bird became ill in a residential area during foraging, the now highly viraemic bird might be too unwell to return to its nightly roost and would remain in the area until its death. Trapping with CO_2-baited

traps was undertaken at the 13 sites. Only one of the six 'control' sites yielded WNV-infected *Cx. tarsalis*. In contrast, six of the seven 'test' sites yielded WNV-infected *Cx. tarsalis* and one yielded WNV-infected *Cx. pipiens*. Nielsen and Reisen (2007) concluded that, during the beginning of the transmission season, mosquito infection rates and, therefore, the risk of human infection are elevated at sites where WNV-infected dead birds are found.

In the Los Angeles area, where American crows were abundant, spatial analyses showed significant clusters of infected dead crows near their roosts. In this area, human and *Culex quinquefasciatus* infection with WNV was significantly greater within the crow clusters than without, indicating the importance of crows in virus amplification. In contrast, in Kern County, where western scrub-jays were evenly distributed, the incidence of human disease was also evenly distributed (Reisen *et al.*, 2006c).

(b) Laboratory estimates of amplifying-host competence

As noted earlier (Section 44.2.2), the 'index of amplifying competence' (C_i) is the product of three factors, such that

$$C_i = s \cdot i \cdot d \qquad (45.10)$$

where s (susceptibility) is the proportion of test birds that become infected upon exposure to infective vectors; i (mean daily infectiousness) is the mean of the proportion of healthy vectors that, day by day, become infected upon exposure to viraemic hosts, and d (duration of infectiousness) is the number of days that a bird maintains an infectious viraemia. The data required for estimates of C_i are obtained through laboratory experiments, either directly as described above, or indirectly using a predetermined threshold titre of 'infectious viraemia', i.e. a supposed minimum titre for infectiousness. For WNV this was taken to be $10^{5.0}$ PFU ml^{-1} serum. The index of amplifying competence is a measure of the sum of the probabilities that a virus will be transmitted from an infected host to a biting mosquito on each of the days that an infectious viraemia is maintained (Komar *et al.*, 2003).

The initial measurements of (so-called reservoir) amplifying competence were undertaken by Komar *et al.* (2003), and remain the only substantial set of competence data. In that investigation, 20 native North American and five exotic bird species were tested for competence as amplifying hosts of the NY99 strain of WNV. Seronegative birds were exposed to infective females of *Culex tritaeniorhynchus*, and the same birds when viraemic were exposed to non-infected mosquitoes. Their indices of amplifying competence were calculated from their mean daily susceptibility to infection by bite, duration of infectiveness and the mean daily infectiveness to mosquitoes. The eight most competent species were all passeriforms, which in the sequence of declining indices of amplifying competence were: blue jay, 2.55; common grackle (*Quiscalus quiscula*), 2.04; house finch (*Carpodacus mexicanus*), 1.76; American crow, 1.62; house sparrow, 1.59; ring-billed gull (*Larus delawarensis*), 1.26; black-billed magpie (*Pica hudsonia*), 1.08; and American robin (*Turdus migratorius*), 1.08. Of the remaining species tested, the values of C_i declined from 0.99 down to 0.03 in ten species, and were zero in seven species.

Few other investigators have measured indices of amplifying competence, but some have coupled indices published by Komar *et al.* (2003) with data of a different sort obtained in the field to achieve a more comprehensive analysis of WNV transmission. An investigation in which a step-flow model was used that ran in 200 daily steps, simulating a 200-day transmission season from 15 March to 1 October, combined field measurements of the sources of blood meals of mosquitoes in Tennessee with published data on vector competence, extrinsic incubation period and daily survival rates from Komar *et al.* (2003) and other sources. The American robin and common grackle were found to be the most important amplifying hosts of WNV (Savage *et al.*, 2007).

Amplifying competence has value as a concept, and the relative values of amplifying competence (so-called reservoir competence) published by

Komar *et al.* (2003) for different bird species are of interest. But the coupling of those values by other investigators of WNV with their own data from wild populations when modelling WNV transmission was inappropriate, because the techniques described in the methods section of that publication were remote from reality. For example: (i) some of the birds had been obtained commercially; (ii) the mosquito vector, *Cx. tritaeniorhynchus*, was not a New World species, and was from a colony obtained from Taiwan in 1997; (iii) the mosquitoes used to infect birds by bite had been infected by intrathoracic inoculation; and (iv) birds were exposed to infective mosquitoes contained within a mesh-sided carton held against their exposed skin.

(c) *Field investigations of overall amplifying competence*

Describing investigations designed to evaluate a different aspects of WNV transmission, Allan *et al.* (2009) introduced two concepts. (i) The 'community competence index', which for any site was the sum of the product of each species' abundance (total number of individuals in the area) and the value of its amplifying competence index (a laboratory measurement). (ii) The 'overall competence' (so-called reservoir competence) of the bird community, being the sum of the community competence indices from all sites (cf. Section 44.2.2).

Three aspects of population biology were thought likely to affect WNV transmission – diversity of bird species, abundance of vector mosquitoes (the numbers trapped in an area) and human density (persons km^{-2}), and their effects were tested on two spatial scales – on a regional scale around St Louis, Missouri, and on the national scale of continental USA. At selected sites around St Louis, estimates were made of bird diversity, human density and vector abundance. Estimates of the 'overall competence of bird communities' used values for the so-called 'reservoir competence' of bird species published by Komar *et al.* (2003). Statistical analysis of data from the St Louis study produced the following findings. The prevalence of WNV in vector mosquitoes was: (i) positively correlated with human density; (ii) positively correlated with the community competence index; and (iii) negatively correlated with bird diversity. Model comparisons suggested that a combination of human density and community competence index was the most important determinants of the prevalence of infected mosquitoes. In contrast, bird diversity and the community competence index were negatively correlated, consistent with the view that less diverse host communities tend to be dominated by the more competent (so-called reservoirs) for zoonotic pathogens (Allan *et al.*, 2009).

The national-scale study resulted in broadly similar patterns to the regional study, i.e. a positive relationship between WNV incidence and human density in 2 out of 3 years; a negative relationship between WNV incidence and bird diversity in all years; and a generally positive relationship between WNV incidence and community competence. However, the correlation between WNV illness and human population density was not significant after controlling for spatial autocorrelation at the national scale, while bird diversity was more strongly correlated with WNV illness than was community competence. The investigators concluded that the effects of bird diversity extend beyond the prevalence of mosquito infection to human disease incidence. Therefore, the loss of bird diversity that tends to accompany urbanization may locally exacerbate the incidence of WNF (Allan *et al.*, 2009).

(d) *Significance of host heterogeneity and host preference*

Transmission of infectious agents within host populations is influenced by many forms of heterogeneity. One consequence of heterogeneity can be an aggregated distribution of infections within a host population, such that a few hosts are frequently or heavily infected, while the majority evade infection or suffer infrequent or light infections (Anderson and May, 1991). Analysis of transmission rates in a number of multi-host diseases revealed that, typically, 20% of the host population contributes at least 80% of the net transmission potential. This statistical pattern,

known as the 20/80 rule, applies to a variety of disease systems. The basic reproduction rate of pathogens, R_0 (Section 45.7.5.a, Eqn 45.6), is affected by the extent of heterogeneity. In a situation in which a host population consists of m subgroups (e.g. m host species), but all other aspects of transmission are identical, R_0 is lower if the host population is homogeneous ($m = 1$) and higher if it is heterogeneous ($m > 1$). The rate of transmission increases as the magnitude of R_0 increases. This has been demonstrated for the transmission of species of *Plasmodium* by *Anopheles*, of *Leishmania* by the sandfly *Lutzomyia*, and of *Schistosoma* by the snail *Bulinus* (Woolhouse *et al.*, 1997).

Regarded as a form of host diversity, host preference among avian hosts was considered to affect the prevalence of WNV in human populations. In Louisiana, where *Cx. nigripalpus* was the most frequently infected mosquito, non-passerine species richness was significantly correlated with both mosquito and human infection rates, whereas there was no significant association between passerine species richness and any measure of infection risk. Ezenwa *et al.* (2006) concluded that non-passerine diversity may play a role in dampening WNV amplification rates in mosquitoes, so minimizing human disease risk.

Several other variables than avian diversity are likely to influence the incidence of WNV infection in human populations. During the period May through to September 2004 at five sites in Maryland and Washington, DC, data were collected on bird abundance, mosquito abundance, vector feeding and the prevalence of WNV. More than 90% of the mosquitoes were *Culex pipiens*. A large proportion of the 174 blood meals taken from engorged mosquitoes and identified by blood meal analysis had come from American robins. Although the robins constituted only 3.7% of total avian abundance, they accounted for 43.4% of mosquito feeding. In contrast, the very much more abundant house sparrows were significantly avoided by mosquitoes and they accounted for only one-quarter as many blood meals (Table 45.34). Thus, the high relative abundance of a host species can be a poor indicator of its relative importance in transmission. Kilpatrick *et al.* (2006a) argued that heterogeneity in host selection by the local mosquitoes resulted in a great heterogeneity in the passage of WNV from its avian to its mosquito hosts.

A separate study was undertaken in 2004 in the same areas, when, again, >90% of host-seeking mosquitoes trapped were *Cx. pipiens* and, during May–June, 51% of all blood meals were taken from American robins although they constituted only 4.5% of the avian community. During the months July to September the abundance of American robins declined dramatically, as they dispersed after breeding, and the proportion of blood meals taken by *Cx. pipiens* on robins also declined, but that coincided with a rise in the use of humans as a blood source by those mosquitoes. During that later period, the total abundance of birds, which was dominated by house sparrows, increased as a result of reproduction. So the shift in the feeding of *Cx. pipiens* from birds to humans was not a result of decreasing avian abundance, but possibly was due to the decline in abundance of American robins, their preferred blood source (Kilpatrick *et al.*, 2006b). In contrast, in north-central California in 2007 and 2008, where >99% of *Cx. pipiens* complex females fed on avian hosts, a higher proportion of avian blood meals was taken by those mosquitoes from American robins during late rather than early summer (Montgomery *et al.*, 2011).

Table 45.34 Characteristics of populations of house sparrows (*Passer domesticus*) and American robins (*Turdus migratorius*) at five sites in Maryland and Washington, DC. (From the data of Kilpatrick *et al.*, 2006a.)

Variable	*House sparrows (mean)*	*American robins (mean)*
Proportion of total avian abundance	55.7 %	3.7 %
Proportion of mosquito blood meals	10.6 %	43.4 %
Initial adult seropositivity in May	19.8 %	56.3 %
Adults seroconverted May to July	11.8 %	42.8 %
Hatch-year birds seroconverted July/August	7.2%	20.0%

(e) Alternatives to biological transmission by mosquito bite

Biological transmission is the transmission of infectious agents between susceptible vertebrate hosts by haematophagous arthropods (Section 41.2.1). A number of forms of transmission of infectious agents between their vertebrate hosts or between the vectors have been described from West Nile virus and other arboviruses.

(1) In birds with acute WNV infections, virus is frequently shed into the cloacal and oral cavities, where very high titres can be detected. When samples were obtained on swabs from the oral cavity or cloaca of wild birds dead for <24 h, and assayed by TaqMan RT-PCR for WNV-specific RNA, the mean log PFU equivalents for oral swabs were: from American crow, 6.6; fish crow (*Corvus ossifragus*), 6.1; and blue jay, 5.7. The mean log PFU equivalents for the cloacal swabs were from: American crow, 6.9; fish crow, 6.0; and blue jay, 6.7 (Komar *et al.*, 2002). This phenomenon was of practical interest as a means of screening dead birds for WNV infection, instead of examining their brain tissue.

(2) It was surmised that WNV might be transmitted to carnivorous birds that fed on infected vertebrates, and to insectivorous birds that fed on infected mosquitoes. In an experimental study, the susceptibility of birds of 25 species to infection with an NY99 strain of WNV by means of oral transmission or supposed contact transmission was examined. Oral transmission was tested either by forcibly placing a suspension of WNV or an infected mosquito at the back of the oral cavity, so stimulating a swallow reflex, or by placing a dead, infected mouse or house sparrow in a cage with a predatory or scavenger bird. Of 15 species of birds that were tested, representing 11 families and seven orders, susceptibility to orally acquired WNV was recorded in only five species. Infection occurred in great horned owls after ingestion of an infected mouse carcass, and in American crows after ingestion of an infected house sparrow carcass, whereas black-billed magpies and a fish crow did not become infected after eating infected house sparrows or infected mice. Infection occurred in the common grackle, house finch and house sparrow after forcible feeding with a suspension of WNV or an infected mosquito.

The possibility of transmission of WNV from infected to uninfected birds by means of so-called contact transmission was tested by caging two birds together, one uninfected and the other having been infected by mosquito bite. Of 18 species of birds tested in this way, representing 12 families and seven orders, transmission to cage mates occurred in only four: ring-billed gull, blue jay, black-billed magpie and American crow, with death rates of 75% to 100%. In the black-billed magpies and American crows, onset of viraemia did not occur until near the time of death of their infected companion. The mode of transmission of WNV from the infected to the uninfected birds was not determined (Komar *et al.*, 2003).

45.7.8 Transmission by migratory birds

The apparently rapid movement of WNV over distances of thousands of kilometres excited interest in its mode of dissemination, with migratory birds considered the most probable vehicle. For certain arboviruses, such as Japanese encephalitis virus, carriage by migratory birds has been considered a possible means of the annual reintroduction to areas of endemicity. After years of investigation, most evidence for transport by migrating birds remains circumstantial. Discussion of this subject requires consideration of a range of topics, including modes of flight, routes of migration and the persistence of viraemias in migrating birds. The single case with direct evidence of transport by migrating birds is the transport of WNV by white storks, which migrate between Africa and Europe, and much of what follows is devoted to that species.

(a) Modes of flight of migrating birds

Of the several modes of flight by birds, three are used by migrating birds – one mode during

migratory flight across oceans, and two during migration overland.

(i) *Dynamic soaring*. Birds that migrate over oceans use a flight procedure termed dynamic soaring in which energy is transferred from horizontally moving air to the bird, so that the energy gained enables it to fly continuously without flapping. Dynamic soaring is possible when the wind speed changes with altitude, a phenomenon termed shear flow (or wind shear), which exists where there are gradients of wind speed in the boundary layer above the ocean surface.

(ii) *Thermal soaring*. Also termed soaring/gliding, this is the main mode of flight of larger birds such as vultures, pelicans and storks, either for patrolling in search of food or travelling long distances. Swifts also make extensive use of thermals, and most birds of prey can be seen soaring in thermals from time to time. Thermals develop where incident solar radiation from the sun passes through the atmosphere and heats the ground. Contact with the warm ground surface heats the lowest layer of air, and the pressure gradient formed causes patches of air to rise through higher elevations as 'thermals'. Large birds gain height by circling within thermals, but the rising air cools with increasing height, and at the elevation at which its temperature matches that of the ambient air, the air ceases to rise and birds change their flight pattern from a vertical soaring to gliding on a straight path. Gliding birds obtain both a vertical or lift force and a forward force from their wings. Lift is generated at right angles to the airflow which, because the bird is descending, comes from slightly below the horizontal. The lift force, therefore, has a forward component that counteracts drag.

(iii) *Flapping flight*. Flapping or powered flight is the familiar flight mode of most birds. When a bird flaps its wings the lift produced is rotated forward so providing thrust, which counteracts drag and increases its speed. The increased speed increases lift, which counteracts the bird's weight, allowing it to maintain height or to climb. It is advantageous for migrating birds to fly at high elevation because their cruising speed increases, resulting in a shorter flight time. A typical cruising height for birds is 2 km. Further, the temperature drops by about 2°C per 1000 feet (305 m), resulting in a reduction of evaporative water loss (Pennycuick, 1969, 1972). Passerines are among birds that use powered flight to fly non-stop for thousands of kilometres when migrating (Curry-Lindahl, 1981).

The fat reserves that a bird accumulates before migration are used as an energy source during flight, and the rate of fuel consumption governs how far the bird can fly. The energy requirements for flapping flight are vastly greater than for soaring/gliding flight, although soaring/gliding flight is not effortless because the pectoralis muscles of a gliding bird must exert a steady force to hold the wings in a horizontal position (Pennycuick, 1972). Generally, thermal soaring flight is advantageous for larger birds, and flapping flight is advantageous for small birds, but even some relatively small birds can save energy by soaring. Flapping produces a much greater flight speed over the ground than soaring. However, the overall speed of migration is affected by the time spent during stopovers for essential energy acquisition. Therefore, the optimal rate of progress is a compromise between the need to cover maximal distances in a given time and the need to save energy and reduce the power needed for flying to a minimum (Hedenström, 1993; Leshem and Bahat, 1999).

The formation of thermals, day by day, is essential for soaring flight. During the early morning, the presence near ground level of a temperature inversion restricts the formation of thermals. Later, clear skies and relative dryness of the ground permit heating of the ground by solar radiation. Once a thermal has broken through any nocturnal inversion it grows in size, strength and vertical extent. At a definite height above the ground, the so-called cloud base, cumulus clouds start to form through the condensation of water vapour carried up by thermals. As solar heating declines during the late afternoon and evening, the thermals become

sparser and weaker near the ground, and eventually die out. In thermal soaring, birds gain height by circling within thermal updrafts, which may be many metres in diameter. They then proceed on their course by gliding while descending until another thermal is encountered (Figure 45.34).

Cloud streets (or thermal streets) are important in the long-distance travel of soaring birds. A cloud street is a long row of cumulus clouds aligned parallel to the direction of wind flow. Under conditions where there are many sources of thermals and a constant wind speed, a series of cloud streets arises in the form of many rows of cumulus clouds that extend downwind, approximately parallel to each other. Below cloud streets the air rises in thermals, while between the cloud streets the air sinks. When cloud streets are present, straight-line soaring becomes possible through thermals that provide less lift than would otherwise be necessary. When a bird flies along a fully developed cloud street it can, by appropriate choice of route, maintain a position in rising air and proceed at any speed at which its sinking speed does not exceed the upward air velocity. Birds sometimes fly straight along cloud streets, without losing elevation, for tens of kilometres (Pennycuick, 1972).

(b) Bird migration between Eurasia and Africa

Investigations into the migration of birds between Eurasia and the African continent have shown a migration from Africa during the spring by many species that pass the summer in northern Europe and Asia, where they breed, and a return migration during the autumn. These birds include both passerines which use flapping flight and large-winged birds such as white storks which employ thermal soaring. Fuller details of the migratory flyways and of their use by migrating birds have been provided by Savage *et al.* (1999). The northern savannahs of sub-Saharan Africa, notably the Sudanese dry and the Senegal-Guinean moist grassland and woodland savannahs are the wintering grounds of the majority of Eurasian species, while the eastern savannahs and highlands, present in much of Kenya, are second in importance. In general, the northwards spring migration of adult birds is rapid, with the birds using fat deposits built up during their stay in Africa to fuel direct flights. It has been surmised that adult birds recently infected with WNV in sub-Saharan Africa could remain viraemic during these northward flights and introduce the virus into southern Europe. The post-breeding southward flights of juveniles and adults may be slower, being interrupted for resting and feeding at intermediate sites. From late March to early May, birds of at least 100 species on a northward migratory flight move through the region of the Danube delta to breed in north-eastern Europe and Asia; from August to October they pass through the Danube delta again on their southward autumn migration. Some species,

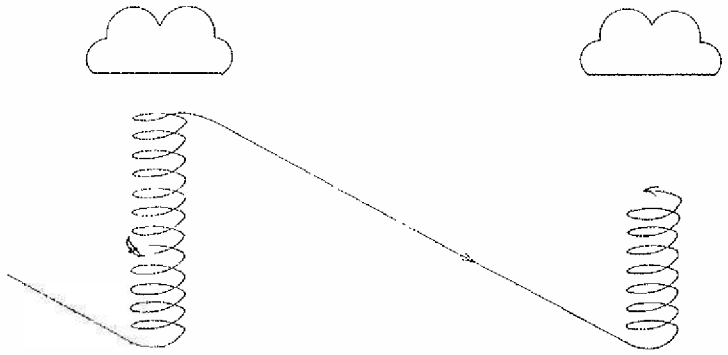


Figure 45.34 Thermal cross-country soaring (From Pennycuick, 1972). In the simplest form of thermal cross-country soaring, a bird gains height by circling in a thermal, then glides on its migratory course, losing the height it had gained, and ascends again in another thermal repeating the sequence. In the best cross-country flying weather the tops of the thermals are marked by small cumulus clouds, not too far apart.

though, fly no further south than the Danube delta, and breed there between mid-May and mid-July. The breeding season of these birds coincides with the build-up of mosquito populations in June and July. *Culex modestus* and *Cx. pipiens*, vectors of WNV, are abundant in the Danube delta. Different genetic forms of *Cx. pipiens* are ornithophagic or zoophagic, and active in sylvatic and urban cycles, respectively, whereas *Cx. modestus* has broad feeding preferences (Savage *et al.*, 1999).

It appears to be widely believed that a condition of viraemia cannot be maintained throughout the duration of a long migratory flight. For example, in discussing the biannual flights of birds between Europe and Africa, Malkinson and Banet (2002) stated that the short period of viraemia in birds, given as about one week, would necessitate a mechanism for virus dormancy and reactivation. However, as noted above, during the spring migration of birds from Africa to Europe the birds use their fat deposits to fuel direct flights, and it was concluded that birds recently infected with WNV in sub-Saharan Africa could introduce the virus into southern Europe. As already noted, the post-breeding return flights of juveniles and adults are slower but the course is overland, permitting resting and feeding at intermediate sites, which could enable a more gradual extension of the distribution of an arbovirus. The presence of WNV in white storks captured in Israel while flying south (Subsection 45.7.8.d below) provides evidence of that.

(c) *Migration of white storks*

White storks are large wading birds, some 100–125 cm in height, with a wingspan of 1.5–2.0 m, a wing area of up to 0.53 m^2, and a mass of 2.3–4.5 kg. Their long and exceptionally wide wings are adapted for soaring flight, by which they can travel long distances with a low expenditure of energy. Measurements of their performance during migratory flight (Table 45.35) show them to be highly capable of soaring/gliding flight. Populations of *Ciconia ciconia ciconia*, the type subspecies of the white stork, breed in western, central and eastern Europe and westernmost Asia, and overwinter in Africa. The European form of the subspecies comprises western populations that winter in West Africa and eastern populations that winter in East Africa. Because of their soaring mode of prolonged flight, white storks avoid large bodies of water, such as the Mediterranean, where no thermals rise. White storks cross from western Europe to Africa at the straits of Gibraltar.

Table 45.35 Flight characteristics of flocks of white storks migrating over Israel, as observed and measured from a motorized glider. (From Leshem and Bahat, 1999.)

Flight characteristics	*Mean value or range*
Climb time within thermals	3.4 min
Thermals exploited per hour	5.7
Altitude of migratory flight	731–463 m
Speed of migratory flight	38.7 km h^{-1}
Daily period of migration	9.0 h
Daily distance of migration	348 km

The following description of the migration of white storks is assembled from reports from ground observers and pilots of motorized gliders who can glide close to the gliding birds. During the southwards autumn migration, flocks of white storks from central and eastern Europe fly first to the Middle East and then follow a route along the Syrian–African Rift Valley, which starts in northern Syria and extends south into Egypt, from where the storks follow the Eastern Rift Valley southwards to spend the northern-hemisphere winter in East Africa, notably Kenya, or southern Africa (Curry-Lindahl, 1981; Van den Bossche *et al.*, 2002; Chernetsov *et al.*, 2004) (Figure 45.35A).

After reaching the Syrian Rift Valley, the white storks fly southwards through the Jordan Rift Valley and past the Dead Sea. As they fly overland east of the Mediterranean Ocean the storks follow one or other of two routes – one more eastern and the other more western. During the southwards migration, between mid-August and mid-September, about 90% of all storks were seen along the eastern axis, a narrow strip of the Jordan Rift Valley, 52–70 km east of the coastline; only a relatively small percentage fly west of this strip. As

evening approached, some stork flocks could be seen landing to roost. Subsequently, the flocks followed the Rift Valley as it turns south-west and traverses the central Negev (Negeb) desert into the Sinai Peninsula and from there into Egypt. During the northwards spring migration, tracked by ground observers, after leaving Sinai most storks were seen to fly along the western axis, a strip 27.5 km wide which runs 10–30 km from the Mediterranean coast and over the western slopes of the Hebron and Samarian mountains. The other migrating white storks followed the eastern axis (Leshem and Bahat, 1999). The route from Russia taken by three young storks equipped with satellite transmitters is plotted in Figure 45.35A.

White storks are gregarious and travel in flocks of 200 to >1000 individuals. Observing white storks from a glider while soaring in company with them over the Serengeti National Park, Tanzania and neighbouring regions, Pennycuick (1972) noted that when flying between thermals the individuals in a flock flew along parallel headings, maintaining a loose formation spread laterally over 200–300 m. The storks appeared to rely on their interactive behaviour patterns to locate lift, rather than responding to the visible conditions as glider pilots can. When part of a flock flew into lift and started to rise relative to the rest, the remainder of the flock converged on those that had started to climb, while those that were rising fastest started to cycle at the centre of the thermal. Flocks of white storks were seen to climb up to the cloud base and

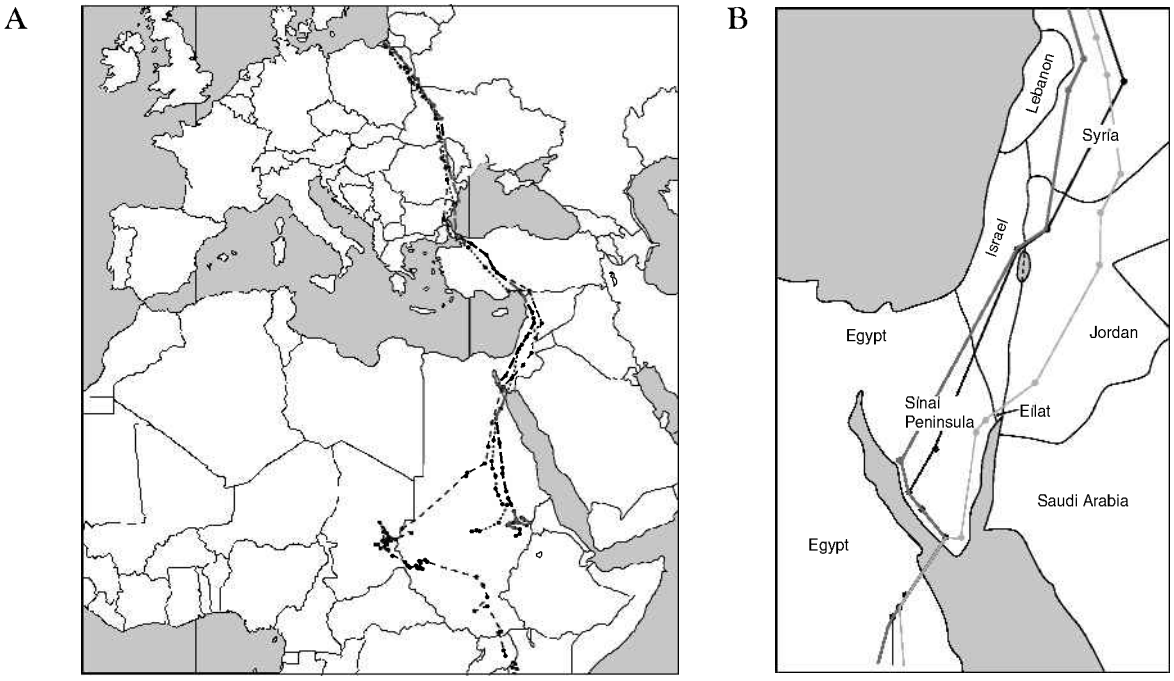


Figure 45.35 **A.** Routes of three young white storks on their migratory flights from the Kalingrad Oblast (an isolated region of Russia, on the Baltic coast situated between Lithuania and Poland). (From Chernetsov et al., 2004.) The birds, from separate nests, were equipped with satellite transmitters; two started their migrations on 10 August and one on 14 August 2000. Their rapid southerly movements ended on 6, 7 and 10 September, respectively, when two birds were in central Sudan and one was in eastern Chad. That bird continued its flight back through southern Sudan and across Uganda into Kenya. (The straight vertical lines over regions of sea are meridians at longitudes 0° and 20°E.) **B.** Redrawn detail of the migratory routes of the three white storks, passing through Syria, Jordan, Israel and the Sinai Peninsula into Egypt. The location of Eilat, where a flock of distressed white storks landed in August 1998, is also shown.

disappear upwards into cloud, but usually they straightened their orientation, and flew just above the cloud base. White storks could proceed under poor conditions, as when overdeveloped cumulus cut off sunshine from the ground. On one such occasion, a flock was followed for 63 km over the Serengeti Plains, and the lift was so weak that the pilot needed to use the engine twice to remain airborne.

(d) Transport of WNV by white storks

White storks migrating from central and eastern Europe frequently stop in the southern part of the Sinai Peninsula before crossing into Africa, many of them in an exhausted condition. During the autumn of 1998 and spring of 1999, among exhausted white storks taken for treatment and subsequent release, 25 individuals with severe injuries were euthanized and samples taken from brain, spleen and serum for virus isolation. Sindbis virus was isolated from 12 of the 25 storks and WNV from one stork (Soliman *et al.*, 2000).

In 1998, unusually strong and hot westerly winds deflected a flock of 1200 migrating white storks eastwards from their route and, in an attempt to correct their direction and reach the Sinai Peninsula, the birds had resorted to powered (flapping) flight. Many of the flock were juveniles less than a year old that had hatched in Europe. They landed on 26 August 1998 at Eilat (29° 33' N, 34° 57' E), at the southernmost tip of Israel, where white storks are only rarely seen (Figure 45.35B). The appearance of the birds showed that they had been under considerable stress and were noticeably weakened. On 28 August, 2 days after the flock landed at Eilat, 13 dead or dying birds were taken for autopsy and four isolates were taken from their brains. (i) The-full length RNA genome of the isolate stork-1998 IS98-STI (AF481864) was sequenced and the complete nucleotide sequence of the greatest part of the viral RNA determined. Sequence analysis of the *E* gene showed almost complete identity with those in isolates from domesticated geese collected in Israel in 1998 and 1999 (goose98, goose99) and a captive white-eyed gull (gull99) (Malkinson *et al.*, 2002). (ii) The nucleotide sequences of Israel 1998-stork (AY0333389) indicated a close relationship with a number of North American isolates collected in 1999–2000 (Lanciotti *et al.*, 2002).

The presence of WNV in the organs of white storks forced to land in Israel during their migratory flight from Europe to Africa suggests that juveniles might have become infected while resting en route in an area such as the Danube delta, with its large passerine avifauna (Subsection 45.7.8.b above). Stops in areas of WNV endemicity during the return migration from Africa to Europe might allow still uninfected white storks to become infected, and so to carry the virus to Europe.

Serological testing of blood samples from 65 white storks, captured between 1 September 1998 and 15 June 2000, provided information on their immune condition. The 65 donor birds comprised 20 taken from the flock that landed at Eilat and that had been placed in a nature reserve to recuperate, 26 migrating across Israel and 19 resident in Israel. Nineteen of the birds (29.2%) were juveniles of <1 year old, of which four (21.0%) carried anti-WNV neutralizing antibodies, while 46 (70.8%) were adults, of which 33 (71.7%) carried neutralizing antibodies. Thus, a total of 56.9% of the birds were seropositive for WNV. The adult storks that were seropositive might have become infected where they had summered in Europe, where they were resident in Israel or where they had overwintered in Africa (Malkinson *et al.*, 2002).

Further information on the transport of WNV to Israel came from investigations into infections in domesticated geese. WNV was isolated from domesticated geese in Israel for the first time in November 1997, when young geese in four flocks presented acute neuromuscular symptoms (Malkinson *et al.*, 1998). The disease reappeared on goose farms in 1998, 1999 and 2000, with death rates as high as 40%. The *E* gene nucleotide sequences of isolates from the brains of a domesticated goose found dead in Israel in 1998, a second goose from Israel in 1999 and a paralysed white-eyed gull (*Ichthyaetus leucophthalmus*) from a breeding colony in the University of Tel Aviv were compared with

those from a white stork from Eilat. The isolates were, specifically, goose98, goose99, gull99 and stork-1998 IS98-ST1 (Table 45.30). Only three nucleotide differences were found between the *E* gene sequences of the four isolates, while the amino acids of the *E* genes of the four isolates were identical. Because the nucleotide sequences of the white stork were almost identical with those from a dead goose in 1988, it was postulated that the epizootic in Israeli geese in 1998 had its origin in Europe, where the virus had been circulating in epidemic proportions since 1996 (Malkinson and Banet, 2002; Malkinson *et al.*, 2002). White-eyed gulls are endemic to the Red Sea, and do not travel much beyond their breeding area.

(e) Possible transport of WNV by birds to and across North America and beyond

The strain of West Nile virus that appeared in New York in 1999 was very similar to one circulating in Israel at that time (Section 45.7.4.b), but how that virus was transported to the western hemisphere has long been problematic. Carriage by infected migratory birds has been largely dismissed for lack of candidates, but importation of exotic birds was regarded as a possibility in light of the infection of exotic birds at two wildlife facilities in New York City during the summer of 1999. Attempts now to explain the passage of WNV across the Atlantic Ocean to North America can only be speculative. The entry of WNV into the USA in 1999 initiated an epornithic in native birds, which extended to an area of 160 km^2 around New York City, as seen by the distribution of dead birds, especially corvids. During 2000, WNV spread to 12 states, and during the years 2001 to 2003 it reached seven provinces of Canada, all but one of the 48 continental USA states, Mexico and other countries in Central America, and the Caribbean (McLean, 2006).

Initial explanations for the mode of spread of WNV invoked migratory birds carrying the virus southwards along the established routes of migration: from the north-eastern states to the south-western states, across the Gulf of Mexico to Central America, and across the Caribbean Sea to South America (Rappole *et al.*, 2000; McLean, 2006). However, the evidence was circumstantial and inconsistent with the nature of the spread of West Nile fever across North America.

Rappole and Hubálek (2003) pointed out that, given the known flight abilities of migratory birds, the virus could be expected to move hundreds of kilometres in a matter of days, whereas in 1999 it moved a maximum of 300 km during the 3 months of known activity, and in 2000 another 400 km during 7 months of known activity. Further, whereas migratory birds generally travelled along a north–south axis, during 2000 when WNV reached 700 km to the south-west it also reached 570 km to the west. Those authors suggested that dispersal movements by house sparrows, a resident species with high seroprevalence for WNV, might be sufficient to account for the long-distance dispersion of WNV, citing the published finding that 25.6% of birds ringed at <1 year of age (n = 755) had dispersed >14 km when next captured as indicating an ability to transport WNV the required 70 km per month. Unfortunately, the mean time to recapture was not stated.

Later, an extensive investigation into the role that migratory birds might play in dissemination of WNV across the USA was undertaken by Dusek *et al.* (2009). Blood samples were taken from birds caught in mist nets at 14 stations along the *Atlantic flyway* and at 7 stations along the *Mississippi flyway*. Blood samples were collected during the periods 2 April to 26 May (spring) and 31 August to 21 October (autumn) to coincide with the peak northward and southward migrations of passerine birds. A total of 13,403 blood samples was collected from 133 species along the two flyways, and all were screened for both West Nile virus and WNV-specific neutralizing antibody. From analysis of the field data and published information on relevant species, the investigators concluded that northern cardinals (*Cardinalis cardinalis*) are possible amplifying hosts of WNV but do not transport it over substantial distances, and that, as migratory birds, grey catbirds (*Dumetella carolinensis*) may play a significant role in the spread of WNV.

In modelling the possible transport of WNV

across the western hemisphere by birds, Rappole *et al.* (2006) developed two stochastic, spatially explicit models, that were 'built on' the life history and ecological data of two representative species, Swainson's thrush (*Catharus ustulatus*), which migrates seasonally between boreal North America and southern Mexico to Argentina, and the house sparrow, a non-migratory resident species. The distance and duration of the migratory flights of Swainson's thrush were known, as were the frequency, direction and distance of dispersal of the house sparrow. It was assumed that, in 1999, Queens, New York, was the original point source of WNV in the New World. The models showed that both rate and pattern of movement observed under the house sparrow–resident bird scenario conformed better to the observed rate and pattern of WNV dispersal than that based on Swainson's thrush movements. The results supported the hypothesis that migrant birds were not responsible for the spread of WNV across North America.

45.7.9 Mammalian hosts

(a) Competence as amplifying hosts

West Nile virus infects wild and domesticated mammals, but the evidence suggests that no or only a few mammals are competent amplifying hosts. Humans, horses and many other mammals that can carry an infection are dead-end hosts because they do not produce an infectious viraemia, and so do not contribute to the transmission cycle (Dauphin *et al.*, 2004). However, as noted below, laboratory observations of viraemia titres indicate that certain sciurids might be competent hosts.

In regions where bird species that are competent amplifying hosts of WNV are present, and where bridge vectors also occur, mammals that are susceptible to WNV and that have been bitten by infective vectors will show as positive in specific serological tests. In an early investigation in Egypt, screening of serum samples for evidence of earlier infections with WNV revealed positivity in a wide range of domesticated animals: camels, cows, water buffalo, donkeys, horses, sheep and goats (Taylor *et al.*, 1956). Serological screening of wild mammals across the USA for past infections with WNV showed that within 7 years of its arrival there in 1999 it had disseminated widely, infecting a monotreme, rodents, carnivores and bats (Table 45.36). Probably, most of those species had been infected by mosquito bites, but the two bat species are insectivorous and might have been infected by ingesting infective mosquitoes (Pilipski *et al.*, 2004).

Cases of equine infection or of seropositivity for WNV have been reported from a number of countries, including France, the USA, Morocco and Italy (Zeller and Schuffenecker, 2004), Mexico (Estrada-Franco *et al.*, 2003), and Cuba (Pupo *et* al.,

Table 45.36 Species of wild mammals caught in the USA and found by serological screening to show evidence of past infections with West Nile virus. Many populations were screened, some with small sample numbers. The records here indicate prevalence rates of more-than-trivial to high, except for the bats with single positive cases only.

Species	*Common name*	*Refs*
Monotreme		
Didelphis virginiana	Virginia opossum	1, 2, 3, 4
Rodents		
Sciurus niger	Fox squirrel	1
Sciurus carolinensis	Eastern grey squirrel	1, 3
Tamias striatus	Eastern chipmunk	3
Rattus norwegicus	Norwegian rat	
Peromyscus leucopus	White-footed mouse	3
Mus musculus	House mouse	1
Carnivores		
Procyon lotor	Coyote	2 ,3, 4
Canis latrans	Raccoon	1, 2
Vulpes vulpes	Red fox	2
Mephitis mephitis	Striped skunk	4
Bats		
Myotis lucifagus	Little brown bat	5
Myotis septentrionalis	Northern long-eared bat	5

References: 1, Root *et al.* (2005); 2, Docherty *et al.* (2006); 3, Gómez *et al.* (2008); 4, Bentler *et al.* (2007); 5, Pilipski *et al.* (2004).

2006). In experimental studies in which 12 horses were each exposed to 12–17 *St. albopicta* infective with WNV, a low-titre viraemia developed in 11 of the 12 horses. Uninfected females that fed on viraemic horses remained negative for the virus, leading Bunning *et al.* (2002) to conclude, despite the small number of bites, that horses are unlikely to serve as amplifying hosts in nature. All domesticated equids appear equally susceptible to WNV, suffering from encephalomyelitis (Kahn, 2005).

The susceptibility of humans to infection with virulent strains of WNV is clear from the epidemics of West Nile fever reported from many countries, and particularly from Egypt, Israel, Romania and the USA. Arboviruses tend to be most virulent in dead-end hosts (Section 44.10.1).

The possibility that species of Sciuridae might be amplifying hosts triggered laboratory experiments in which wild-caught sciurids were held captive and infected with WNV by mosquito bite or syringe inoculation. Mean viraemias of $>10^{3.0}$ and $>10^{4.5}$ developed on days 2 to 4, but no viraemia developed in the only squirrel with pre-existing antibodies to WNV (Root *et al.*, 2006). In a comparable study, also with fox squirrels, the viraemias that developed in individuals subjected to infective mosquito bite or syringe inoculation were of essentially the same titre. The combined data showed mean titres on days 2–5 post-infection of $10^{4.4\ (4.0–4.8)}$, $10^{5.3\ (5.0–5.6)}$, $10^{4.4\ (3.9–4.9)}$ and $10^{2.7\ (2.0–3.4)}$ PFU ml^{-1} (95% c.i.) The highest individual serum titres following mosquito bites ranged from $10^{5.1}$ to $10^{5.3}$, and on average the serum titre persisted at $\leq 10^{5.1}$ PFU ml^{-1} for 1.6 days. Rates of disseminated infection in *Cx. pipiens* that had fed on fox squirrels (*Sciurus niger*) with serum titres of $10^{4.4 \pm 0.1}$ or $10^{5.5 \pm 0.1}$ PFU ml^{-1} ± s.e. were 13% and 89%, respectively (Platt *et al.*, 2008).

Intramuscular inoculation of eight eastern chipmunks (*Tamias striatus*) with $10^{1.5}$ to $10^{5.7}$ PFU ml^{-1} WNV resulted in mean viraemia titres on days 1–3 post-inoculation of $10^{3.9\ (3.3–4.5)}$, $10^{6.7\ (6.4–7.0)}$ and $10^{5.8\ (4.7–7.5)}$ PFU ml^{-1} (95% c.i.), respectively. The estimated mean number of days that serum titres remained at either $\geq 10^{4.8}$ or $10^{5.6}$ PFU ml^{-1} were 1.7 and 1.4, respectively (Platt *et al.*, 2007).

The eastern cottontail rabbit (Leporidae: *Sylvilagus floridianus*), which ranges throughout most of the USA east of the Rocky Mountains, was considered a possible amplifying host of WNV although there were no field data. Seven cottontail rabbits infected with WNV by the bites of one to nine infective mosquitoes developed mean viraemias that declined from a 'cell culture infective dose 50% ($CCID_{50}$)' of $10^{-4.8}$ on day 2 post infection to one of $10^{-4.1}$ on day 4. The viraemia titres in eight rabbits infected by subcutaneous injection were slightly lower. For all 15 rabbits, the mean durations of viraemia titres of $\geq 10^{4.3}$ and $>10^{5.0}$ $CCID_{50}$ were 2.2 and 1.0 days, respectively. The estimated MIRs of *Cx. pipiens* feeding on rabbits with viraemias of 5.0 to <5.5 and of 5.5 to ≥5.8 were14/1000 and 27/1000, respectively. For *Cx. salinarius*, the equivalent MIRs were 28/1000 and 22/1000 (Tiawsirisup *et al.*, 2005).

(b) Shedding of virus

In birds with acute WNV infections, virus is frequently shed into the cloacal and oral cavities, where very high titres can be detected (Section 45.7.7.e). Comparable shedding can occur in infected mammals.

When captive fox squirrels had been infected by needle inoculation or mosquito bite, WNV was recovered from both oral and rectal cavities of some 40% to 50% of squirrels on day 2 post-exposure, and from both cavities of 80–100% of squirrels on days 3 to 7 post-exposure, after which the recoveries declined rapidly. However, WNV was recovered from the rectal cavity of ~40% of squirrels on day 11, while from one individual it was recovered from the oral cavity on day 17, and from another on day 22. The amounts of WNV recovered from oral swabs ranged from $10^{1.9}$ to $10^{2.9}$ PFU (n = 4). During the first 7 days post-exposure, and first on day 3, WNV was present in 25% of urine swabs (n = 28) collected opportunistically from five squirrels. From two of those squirrels, WNV was recovered from urine swabs on

day 11 or 17. The largest amount of WNV recovered from a urine swab was $10^{3.1}$ PFU (Platt *et al.*, 2008). Manual administration of a WNV suspension into nine anaesthetized fox squirrels led to viraemias in five, all of titres known to be sufficient to infect blood-feeding mosquitoes (Tiawsirisup *et al.*, 2010).

45.7.10 Mosquito vectors

(a) Main vectors of WNV

The identities of putative vectors of WNV found during early investigations into the occurrence and transmission of West Nile virus in Egypt were a guide to the involvement of species of *Culex*. WNV was isolated from wild-caught *Cx. pipiens*, *Cx. antennatus* and *Cx. perexiguus*, species that exhibited almost all of the characteristics required of main vectors (cf. Section 41.1.3). In the laboratory, females of those species fed when exposed to viraemic birds, and after a period of extrinsic incubation could transmit the virus to uninfected birds (Table 45.31). When from one to ten females of *Cx. pipiens* or *Culex perexiguus* that were infective with WNV fed on birds, one bite was as effective as many in infecting the recipient host (Work *et al.*, 1955).

WNV has been isolated from wild-caught mosquitoes of at least 11 genera. The isolation of an arbovirus from wild mosquitoes indicates no more than that those individuals had ingested blood from viraemic hosts (possible infection by vertical transmission apart). So the description, in a CDC report, of 64 species that had been captured in the USA during the years 1999 to 2008 and that had tested positive for WNV as 'medically important' was premature. West Nile virus is transmitted predominantly by mosquitoes, but it has been isolated from ticks in Africa, Europe and Asia. However, Lawrie *et al.* (2004) found that an ixodid tick was unable to maintain the virus, and only poor transmission was achieved through an argasid tick.

Blitvich (2008) named ten species from different geographical regions as main vectors of WNV to human hosts, but, here, *Cx. modestus* is also treated as a main vector in the Danube delta and the Camargue region of France; the characteristics of those 11 species are summarized in Table 45.37. Of those species, ten are members of the subgenus *Culex* (*Culex*), while *Cx. modestus* is a member of *Culex* (*Barraudius*).

(b) Species of the **Culex pipiens** *subgroup*

Culex pipiens and *Cx. quinquefasciatus* are responsible for much of the transmission of WNV throughout its range. Because of the extent and the variety of their interactions with the avian and mammalian hosts of WNV, the descriptions of mosquito transmission of WNV will focus on those two species. Both are members of the Pipiens Complex (Harbach, 2010c).

Culex (*Culex*):
Pipiens Group:
Pipiens Subgroup:
Cx. globocoxitus
Pipiens Complex:
Cx. australicus
Cx. pipiens: Subspecies: *Cx. p. pipiens*,
Cx. p. pallens
Cx. quinquefasciatus

Of these species, *Cx. australicus* and *Cx. globocoxitus* are essentially limited to Australia, and at present are not exposed to West Nile virus. The subspecies *Cx. p. pallens* occurs in Japan, China and Korea. It is anautogenous, bird biting, hibernates in the adult female stage, and at present is not exposed to WNV (Kasai *et al.*, 2008). The presence of a distinct microsatellite signature in *Cx. p. pallens* indicated restricted gene flow between western and eastern populations of *Cx. pipiens*, supporting the existence of the two subspecies (Fonseca *et al.*, 2009). *Culex p. pipiens* is widely distributed through the temperate zones of both hemispheres, and extends into the subtropical zones. *Culex quinquefasciatus* has a cosmotropical distribution, and extends far into the subtropical zone of the southern hemisphere.

Culex p. pipiens and *Cx. quinquefasciatus* are very important vectors of WNV. It is owing to behavioural and physiological differences between

Table 45.37 Characteristics of species of *Culex* (*Culex*) that were identified by Blitvich (2008) as the most important vectors in the transmission of West Nile virus in different parts of the world. *Culex modestus* of *Culex* (*Barraudius*) has been added. The species named *Cx. univittatus* by Blitvich (2008) is here named *Cx. perexiguus*, Harbach (1999) having reported the previous misidentification of populations in certain geographical regions. To show the taxonomic affinities of the species, their assignment to subgenera, species groups and subgroups are indicated.

Culex *species*	*Distribution*	*Host preferences*	*Other characteristics*
Cx. annulirostris [6]	Australasian Region, Indonesia, Philippines	Birds, notably waterbirds	In Australia main vector of Kunjin virus
Cx. antennatus [2]	Middle East and sub-Saharan Africa	Mammals, especially cattle and humans; birds	Various larval habitats
Cx. modestus [8]	Palaearctic Region	Birds	Develop in wetland pools
Cx. perexiguus [4]	Arid areas in eastern Africa and Mediterranean subregion	Mainly birds; humans	Main vector in Israel; various larval habitats
Cx. pipiens, pipiens ecotype [1]	Temperate parts of Palaearctic and Nearctic Regions	Birds	Above-ground aquatic habitats; anautogenous
Cx. pipiens, molestus ecotype [1]	Scattered populations through Palaearctic and Nearctic Regions	Mammals	Below-ground aquatic habitats; autogenous
Cx. quinquefasciatus [1]	Subtropical and tropical zones	Opportunistic on mammals and birds	Above-ground aquatic habitats; anautogenous
Cx. restuans [5]	Central and eastern USA; northern Neotropical Region	Birds >> mammals	Can be sympatric with *Cx. pipiens*
Cx. pseudovishnui [7]	Oriental Region	Cattle > pigs	Develop in paddy fields
Cx. tritaeniorhynchus [7]	Oriental, Palaearctic and Afrotropical Regions	Cattle > pigs	Develop in paddy fields
Cx. vishnui [7]	Oriental Region	Cattle > pigs >> humans, chickens	Develop in paddy fields
Cx. tarsalis [3]	Southern Canada to northern Mexico	Birds and mammals	Main vector in southern part of WNV distribution

Subgenus *Culex* PIPIENS GROUP: [1], Pipiens Subgroup; [2], Decens Subgroup; [3], Tarsalis Subgroup; [4], Univittatus Subgroup; [5], Restuans Complex. SITIENS GROUP: [6], Sitiens Subgroup; [7], Vishnui Subgroup; [8], **Subgenus *Barraudius***.

them, and to their different distributions, that the epizootiology and epidemiology of WNV differ markedly in different zoogeographical regions.

(c) *Ecotypes of* Cx. p. pipiens

Populations of the subspecies *Cx. p. pipiens* occur in two types, which differ slightly but inconsistently in their morphological characteristics (Harbach *et al.*, 1985), but which, because of differences in particular behaviour patterns and physiological characteristics, have distinct ecologies. Certain authors have ranked them as genetic 'forms' or 'biotypes', e.g. the 'pipiens biotype' and the 'molestus biotype'; other authors consider them to be of distinct species – the subspecies *pipiens* of *Cx. pipiens* L. and *Cx. molestus* Förskal. The descriptive term 'ecotype', used by Chevillon *et al.* (1995), is more appropriate and is used here. By definition, an ecotype is a locally adapted population or infraspecific group having distinctive characters which result from the selective pressures of the local environment or microenvironment (Lincoln *et al.*, 1998).

The pipiens ecotype has the following characteristics: (i) all life stages are epigeal, i.e. occur

above the ground surface, where the aquatic stages develop in bodies of fresh water; (ii) the adults are eurygamous (requiring a large space in which to mate by swarming); (iii) the adult females are ornithophilic; (iv) they are anautogenous (requiring a blood meal to develop a batch of oocytes to maturity); and (v) they hibernate during the winter. Contrasting characteristics of the molestus ecotype are: (i) all life-cycle stages are hypogeal, i.e. occur below ground, and in spaces that have only limited access and egress for adults, the larvae developing in organically polluted water; (ii) the adults are stenogamous (capable of mating without swarming, in a very small space); (iii) the adult females are anthropophilic; (iv) they are autogenous (not requiring a blood meal to develop the first batch of oocytes to maturity); and (v) adults remain active through the winter in sheltered, subterranean biotopes, often in total darkness, that maintain a sufficient temperature to permit continuous breeding. Characteristically, hypogeal sites used by the molestus ecotype are man-made, e.g. flooded basements, underground railway tunnels, below-ground drainage sumps, sewage works, etc.).

A field study of populations of *Cx. p. pipiens* near Barcelona showed that the populations of hypogeal sites that were only 'partly enclosed', such as underground drains and cesspits (so-called intermediate sites), did not differ from populations from epigeal sites in expression of the characters stenogamy/eurygamy and autogeny/anautogeny. Chevillon *et al.* (1995) concluded that two sets of habitat can be distinguished as significantly distinct: (a) hypogeal sites that are almost totally enclosed; and (b) 'intermediate' sites together with fully epigeal sites.

Inbreeding and cross-breeding experiments between London populations of the molestus and pipiens ecotypes, which involved mating within small cages, demonstrated the significance of mating behaviour. All crosses between males and females from different hypogeal (molestus ecotype) populations resulted in viable F_1 progeny, but no crosses between epigeal (pipiens ecotype) males and autogenous (molestus ecotype) females resulted in any offspring, confirming that the epigeal (pipiens ecotype) males were not stenogamous (Byrne and Nichols, 1999).

(d) Gene flow and the differentiation of ecotypes

Molecular-genetic analytic techniques have been used to differentiate the two ecotypes. Fonseca *et al.* (2004) used eight highly polymorphic microsatellite markers to fingerprint specimens collected from hypogeal and epigeal populations in different zoogeographical regions. Autogenous mosquitoes from underground microhabitats in different geographical regions were genetically similar to one another, but genetically differentiated from mosquitoes from above-ground habitats even when sympatric or geographically close, as was apparent from the standard distance measurements and the allelic frequency distributions. The two ecotypes were considered distinct species.

Later, Bahnck and Fonseca (2006) developed a more rapid assay based on variation in the microsatellite CQ11, which differentiated between the two genetic forms, and named them 'form pipiens' and 'form molestus'. Shaikevich (2007) reported that the 'pipiens form' and the 'molestus form' of *Cx. p. pipiens* could be distinguished by PCR-RFLP of the 5' end of the mtDNA *COI* gene.

A number of investigators have estimated the extent of gene flow between hypogeal and epigeal populations of *Cx. p. pipiens*. As cited above, Fonseca *et al.* (2004) found no evidence of gene flow. In contrast, from genetic studies of populations of *Cx. p. pipiens* around Barcelona it was estimated that all gene flows between below-ground and above-ground habitats were >1, indicating that 'migration' was too strong to allow for the fixation of alternative alleles in the different habitat types by drift alone (Chevillon *et al.*, 1995).

In a different approach to the problem, assessments were made of two genetic features. (i) Much higher genetic distances were found between paired local populations of the pipiens and molestus ecotypes than between geographically separated populations of either ecotype. (ii) When F_{st} (fixation index) values were calculated to estimate gene flow between populations, substantial restric-

tion was found on gene flow between paired local populations of the two ecotypes, whereas restriction on gene flow between geographically separated populations of the individual ecotypes was much less. That could have been due to behavioural differences between the two ecotypes (Weitzel *et al.*, 2009).

In the Comporta region of Portugal (at 38° N, 46° W), above-ground breeding populations of both *Cx. p. pipiens* ecotypes were present, distinguishable by microsatellite analysis. Populations of the pipiens ecotype were eurygamous and anautogenous; those of the molestus biotype were stenogamous and autogenous. Of the limited gene flow between them, more molestus ecotype genes introgressed into the pipiens ecotype than vice versa (Gomes *et al.*, 2009).

(e) Culex pipiens pipiens *in the Palaearctic and Nearctic Regions*

The populations of *Cx. p. pipiens* in the Palaearctic and Nearctic Regions differ substantially, both genetically and phenotypically. In what was intended as a genetic comparison of above-ground and below-ground populations from different countries, an unrooted distance tree was produced based on proportion of shared alleles. The tree comprised three distinct clones: underground populations from Europe and the Middle East clustered separately from European above-ground populations, and North American populations clustered separately from both, supposedly because of hybridization. Populations from northern Europe that differed in behaviour and physiology had unique microsatellite fingerprints, with no evidence of gene flow between them. In the USA, hybrids between the two forms were said to be ubiquitous (Fonseca *et al.*, 2004). The northern Palaearctic and Nearctic populations of *Cx. p. pipiens* differ markedly in ways that affect their role as vectors of West Nile virus, and sufficiently greatly for it to be necessary to describe them separately.

In the more northerly latitudes of the **Palaearctic Region**, populations of the pipiens ecotype are widespread in rural areas, whereas populations of the molestus ecotype occur in scattered urban locations over the same area. The physical separation of the habitats and the different mating behaviours of the two ecotypes tend to limit interbreeding. At these latitudes, the ornithophilic females of the pipiens ecotype are excellent enzootic vectors of West Nile virus, circulating the virus among birds; in contrast, females of the molestus biotype are anthropophilic and can infect humans.

In the warmer Mediterranean subregion of the Palaearctic Region, populations of the pipiens and molestus ecotypes are present, but other populations have been described in which certain characters of the molestus ecotype were found in populations that developed in surface waters. These populations might be eurygamous or stenogamous; they usually showed a low rate of autogeny; the females were strongly attracted to humans and other mammals but more weakly to birds; and they were able to breed through the winter months. They were thought not to be hybrid populations resulting from cross-breeding between the pipiens and molestus ecotypes. Early descriptions came from Arles and Antibes in the south of France, and Cairo, Algiers and Tunis in North Africa (Roubaud, 1933; Knight and Abdel Malek, 1951; Roubaud and Ghelelovitch, 1956).

In southernmost Europe, Fonseca *et al.* (2004) identified two populations with a few hybrid individuals, and cited an earlier report by Urbanelli *et al.* (1981) of allozyme-based studies which revealed the existence in Italy of populations with a mix of the two ecotypes but a very low rate of hybridization (1%), possibly because of their different mating behaviours.

Culex pipiens (*Cx. p. pipiens*) is widespread and abundant in much of the **Nearctic Region**. It occurs as far south as 39° N, between 39° N and 36° N overlapping with populations of *Cx. quinquefasciatus*. To examine the relationship between North American populations and populations from Europe and elsewhere, a multilocus genotype analysis was performed on individuals from 33 populations. Three, most likely, distinct

genetic clusters were identified: cluster A, the great majority of European above-ground populations; cluster B, autogenous specimens; and cluster C, *Cx. quinquefasciatus*. All US populations of *Cx. pipiens* formed a group set apart from the three 'distinct' clusters, and all included hybrids of the two ecotypes, pipiens and molestus. To hybridization between 'bird biters' and 'human biters', Fonseca *et al.* (2004) ascribed important differences of epizootiology and epidemiology between US and European populations of *Cx. pipiens*, notably in their roles as vectors of WNV.

It should not be overlooked that no comparable investigations have been undertaken in the more northerly latitudes of North America, in which distinct populations of the pipiens and molestus ecotypes might be expected. None of the specimens examined by Fonseca *et al.* (2004) was from a location north of 43° N.

During three successive years in the 1960s, Andrew Spielman investigated autogenous and anautogenous populations of *Cx. pipiens* that were in close proximity in an urban part of the city of Boston, Massachusetts (c. 42° N). Autogenous larvae were abundant in enclosed sites, as were anautogenous larvae in sites that provided free access and egress. As the day length shortened after mid-August, anautogenous larvae disappeared and the adult females were conditioned to enter hibernation, but the abundance of autogenous larvae increased until mid-October. Cross-mating between the two forms occurred occasionally in late summer, but effectively they were reproductively isolated. The anautogenous females fed mainly on birds. The autogenous females hardly ever took blood meals, suggesting low survival after the first gonotrophic cycle. Similar autogenous and anautogenous populations of *Cx. p. pipiens* occurred as far south as 33° N (Spielman, 1964, 2001).

(f) Culex quinquefasciatus

At southerly latitudes of the Palaearctic and Nearctic Regions, *Culex quinquefasciatus* is a common domestic and peridomestic species. Larval development takes place mainly in polluted water, but also in fresh-water and brackish pools; mating occurs in swarms in large open spaces (eurygamy); females feed opportunistically on birds or mammals; a blood meal is required before all ovarian cycles (anautogeny); and adult females are incapable of diapause. In the western Palaearctic Region there are few interfaces between the populations of *Cx. pipiens* and *Cx. quinquefasciatus*, but introgression occurs where the species overlap in southern Iraq and across the Arabian Peninsula (Harbach, 1988). In North America, on the basis of anatomical and allelic characteristics, it was concluded that only populations of *Cx. p. pipiens* occur north of about 39° N, and only populations of *Cx. quinquefasciatus* south of about 36° N. Between 36° N and 39° N there is a zone of intergradation, in which populations of three types occur: (i) *Cx. pipiens*; (ii) *Cx. quinquefasciatus*; or (iii) intergrades, which possess intermediate characters or traits (Barr, 1957; Cheng *et al.*, 1982).

Populations of intergrades in the intergradation zone are not F_1 hybrids between *Cx. p. pipiens* and *Cx. quinquefasciatus* but appear to be stable breeding entities (Tabachnick and Powell, 1983). The intergradation zone generally is a north–south cline of decreasing *Cx. p. pipiens* and increasing *Cx. quinquefasciatus* populations, but it is not uniform. In the northern part of the Central Valley of California, the cline is reversed, possibly owing in part to a reversal of temperature gradients where cool sea air penetrates into the valley (Tabachnick and Powell, 1983; Urbanelli *et al.*, 1997). From the morphological characteristics and electrophoretic profiles of *Cx. pipiens* and *Cx. quinquefasciatus*, Cornel *et al.* (2003) concluded that in California there is incorporation of genes from the gene pool of each species into that of the other, i.e. genetic introgression, whereas in South Africa populations of the two species are genetically distinct and behave accordingly.

(g) Roles of Culex p. pipiens *and* Culex quinquefasciatus *as vectors of WNV*

In the Palaearctic Region, females of the pipiens ecotype of *Cx. p. pallens* are ornithophilic, while

those of the molestus ecotype are anthropophilic. Within the range of WNV distribution in that region, West Nile disease may occur commonly in susceptible bird species, but only infrequently in human populations, which become infected only upon exposure to infective populations of the below-ground molestus ecotype. The much wider distribution of West Nile disease in North America, in both susceptible bird species and humans, has been ascribed to the presence of hybrids between the pipiens and molestus ecotypes in most populations, and also to the greater virulence of North American strains of WNV.

Descriptions of an outbreak of West Nile disease in Romania during 1996 provide an informative if broad-brush picture of a major epidemiological event under the ecological conditions in parts of the Palaearctic Region. Passerine birds occur in abundance across the Danube delta, and are subject to infection with WNV by the bites of infective females of the pipiens ecotype of *Cx. p. pipiens* and of *Cx. modestus*. Many migratory birds rest in the delta while *en route* to their final destination. While there they are at risk of infection, and migrants of the same species, infected elsewhere, may introduce WNV to the delta on their return flights (Savage *et al.*, 1999).

In Bucharest (at 44° N) during the mid-1970s, a construction programme began in which many single-family dwellings were destroyed and replaced with tall apartment buildings known as block-houses, which were built over a much older drainage system designed to remove rainwater. By 1996, in some blockhouses, both water pipes and sewage pipes had deteriorated to the extent that basements were partially flooded with a mixture of fresh water and raw sewage. Aquatic stages of the molestus ecotype were present in some flooded basements, giving rise to adults which rested in hundreds and sometimes thousands on the ceilings of the hallways above. Interspersed between some block-houses were single-family homes, with poultry sheds containing amplifying hosts of WNV such as domestic fowls, and container habitats occupied by larvae of the pipiens ecotype.

The pre-epidemic bird population in Bucharest was ample in size and susceptible to infection with WNV; in fact, rates of WNV seroprevalence in domestic fowls were comparable in urban Bucharest and at rural sites. Both the molestus and pipiens ecotypes of *Cx. pipiens* were present in Bucharest, where they occupied different ecological niches. Isolates of WNV were obtained from females of the molestus ecotype. The earliest confirmed cases of the 1996 epidemic in Bucharest presented on 15 July 1996, the epidemic peaked in early September, and the last confirmed cases were reported 3 weeks later. The outbreak in Bucharest produced at least 393 hospitalized cases, all with neurological disorders, and a 4.3% death rate (Tsai *et al.*, 1998; Savage *et al.*, 1999; Campbell *et al.*, 2001).

In the Mediterranean subregion of the Palaearctic Region, where some above-ground populations of *Cx. p. pipiens* show certain characteristics of the molestus ecotype, the presence of mammal-biting females may account for the infection of horses and humans with WNV. In Egypt, a wide range of domesticated animals is subject to infection with WNV (Taylor *et al.*, 1956).

In northerly latitudes of the Nearctic Regions, populations of both the pipiens and molestus ecotypes of *Cx. p. pipiens* may occur, as in Boston, Massachusetts (Subsection 45.7.10.e above). More widely though, populations of *Cx. p. pipiens* include hybrid individuals, with the possibility of attacks on birds and humans leading to enzootic infections with WNV in wild birds and sporadic infections in humans.

How far north populations of *Culex quinquefasciatus* extend in different regions has an important effect on transmission of WNV, because the females feed on both birds and mammals. In the Nearctic Region, the interface between *Cx. pipiens* and *Cx. quinquefasciatus* is the intergradation zone, between 36° N and 39° N. From there southwards, *Cx. pipiens* is absent and *Cx. quinquefasciatus* becomes a main host of WNV. In the western Palaearctic Region, populations of *Cx. pipiens* extend only towards the southern boundary of the Mediterranean subregion (Harbach, 1988). In Africa, there is a gap of some degrees latitude

between the southern limit of *Cx. pipiens* and the northern limit of *Cx. quinquefasciatus*. In South Africa, sympatric populations of the two species overlap but remain genetically distinct (Jupp, 1978).

In the western Palaearctic Region, most strains of WNV are relatively benign, and records of human cases of WNF suggest a slow rate of dissemination across the region. However, the mild cases of WNF in Israel during 1951 changed to severe cases in Central Europe in the 1990s. In contrast, from its first appearance in the USA, the strains of WNV were virulent, which may account in part for the rapid dissemination of WNV from New York in 1999 to all 48 contiguous states and seven Canadian provinces by 2004.

(h) Possible transport of WNV by mosquitoes across the USA

The westward movement of WNV across the USA was possibly aided by the random dispersive movements of infected resident birds (Section 45.7.8.e). The possibility of a similar contribution by infected mosquitoes was investigated by Venkatesan and Rasgon (2010). The species largely responsible for WNV amplification in the western USA is *Cx. tarsalis*. Analysis of 12 microsatellite loci was used to elucidate genetic structure and barriers to gene flow in 20 populations of *Cx. tarsalis* across 16 states. That revealed the presence of three broad clusters of populations with barriers to gene flow, which possibly were isolated by geographical features known to affect the dispersal of *Cx. tarsalis*. However, over large portions of the West Coast and within the Great Plains region, small genetic distances among populations within clusters indicated that gene flow was not obstructed. The movement of genes from one population to another indicates interbreeding following the migration of individuals, and if the migration of infected individuals then that of the pathogenic agent also. The observed spatial pattern of genetic clustering in *Cx. tarsalis* mirrored the pattern of invasion of WNV across the western USA, raising the possibility that movements of those mosquitoes had assisted the dispersal of WNV. Overall, gene flow in *Cx. tarsalis* appeared to be extensive, potentially mediated by movement of mosquitoes among neighbouring populations, and hindered in geographically limited parts of its range.

Mark–release–recapture studies in California showed that female *Cx. tarsalis* can travel several kilometres a night on consecutive nights while host seeking or seeking oviposition sites, suggesting that the spread of WNV by the flight of those vectors might be possible (Reisen and Reeves, 1990; cited by Venkatesan and Rasgon, 2010). Given the propensity of *Cx. tarsalis* to move and the evidence of high gene flow across large geographical areas, Venkatesan and Rasgon (2010) surmised that it might have been involved in the dispersal of WNV in the western USA.

46

Pathogenic and symbiotic bacteria

Relatively few species of bacteria are known to be pathogens or symbionts of culicids, but certain of those are of economic importance or academic interest. Take two examples. During sporulation, *Bacillus thuringiensis* and *Lysinibacillus sphaericus* secrete crystalline, proteinaceous, parasporal bodies containing toxins that are lethal to insects, and these have been developed as bioinsecticides. The intracellular symbiont *Wolbachia pipientis*, which infects a wide range of arthropod hosts, modifies their reproduction by a variety of mechanisms to its own advantage. Among mosquitoes, it infects virtually all populations of *Culex pipiens* and *Culex quinquefasciatus* and populations of certain *Stegomyia* species, and by affecting karyogamy determines the possibility of cross-breeding between populations.

46.1 CLASSIFICATION AND SPECIES DEFINITION

46.1.1 Classification

Classifications of living organisms have at their base three taxa, sometimes ranked as domains: the Archaea, Bacteria and Eucarya. Commonly, the unicellular Archaea and Bacteria are grouped together in the taxon Prokaryota, while the multicellular Eucarya are given equal rank as the taxon Eukaryota; these two taxa are commonly referred to as prokaryotes and eukaryotes. This division of living organisms into prokaryotes and eukaryotes has attracted criticism. Pace (2009) pointed out that the 'root' of the universal tree fundamentally separates Bacteria from Archaea and that there is no phylogenetically based grouping that contains bacteria and archaea; and so he concluded that there 'can be no such thing as a prokaryote in any phylogenetic classification'. On those grounds, he recommended that the term prokaryote be discontinued, while accepting that this will be hard to implement because internationally agreed rules regulate the nomenclature of prokaryotes. Use of the term prokaryote is continued in this volume.

The scientific names of prokaryotes up to the rank of Class are regulated by the *International Code of Nomenclature of Prokaryotes* (De Vos and Trüper, 2000). Published names that conform to the Rules of Nomenclature are considered valid, and when approved are published in the *List of Prokaryotic Names with Standing in Nomenclature* (Euzéby, 2011). Some authors italicize names of higher rank than

genus, a practice that is formally acceptable but not required. Taxonomic ranks above species are not defined but can be considered abstract entities (Rosselló-Mora and Amann, 2001). If the informal term 'bacteria' was to be replaced by the term 'prokaryotes', the name Bacteria would still continue in use as the formal name of one of the prokaryote domains (De Vos and Trüper, 2000).

Prokaryotes share a number of characteristics. Nuclear membranes are absent, which allows the coupling of transcription and translation. Most of their genomic DNA is contained in a single circular chromosome, but up to about 4% is contained in plasmids which replicate independently. There is no internal cytoskeleton or endomembranes, and an absence of mitosis and of true sex. The bounding cytoplasmic membrane is composed of lamellae, tubules and other cytoplasmic intrusions; it is multifunctional, and membrane transporters assimilate dissolved nutrients from the environment (Whitman, 2009). Other characteristics distinguish bacteria from archaea. Whereas bacteria use sigma factors to control the initiation of transcription, archaea use TATA-binding proteins. Bacteria wrap their DNA in a variety of basic proteins, whereas many archaea use histones. Bacteria use ester-linked lipids in structuring their membranes, whereas archaea use ether-linked lipids (Pace, 2009).

The remainder of this chapter predominantly concerns species of Bacteria, and, because Archaea have no known interactions with mosquitoes; further reference to Prokaryotes is avoided except where it is a cloak for Bacteria. The updated *Bergey's Manual of Systematic Bacteriology*, Vol. 2(A) (Garrity, 2005), provides a classification of prokaryotes based principally on intensive sequence analysis of 16S rRNA. Because of its widespread acceptance, the classification of the Bacteria given in the updated *Bergey's Manual* is described below, but with strong reservations: it is unwise in principle to base too much on a single molecule (rRNA); the monophyly of groups that are robustly monophyletic by classical morphological criteria has been over-confidently denied; and much of the deep structure of early rRNA trees has been proved artefactual (Philippe *et al.*, 2000; Cavalier-Smith, 2002; Roger and Hug, 2006). The following outline classification from *Bergey's Manual* is limited to lineages that lead to the five genera of interest here (*Bacillus*, *Lysinibacillus*, *Clostridium*, *Spiroplasma* and *Wolbachia*):

Domain: Bacteria
- Phylum: Proteobacteria
  - Class: 'Alphaproteobacteria'
    - Order: Rickettsiales
      - Family: Anaplasmataceae
        - Genus: ***Wolbachia***
- Phylum: 'Firmicutes'
  - Class: 'Clostridia'
    - Order: Clostridiales
      - Family: Clostridiaceae
        - Genus: ***Clostridium***
  - Class: Mollicutes
    - Order: Entomoplasmatales
      - Family: Spiroplasmataceae
        - Genus: ***Spiroplasma***
  - Class: 'Bacilli'
    - Order: Bacillales
      - Family: Bacillaceae
        - Genera: ***Bacillus, Lysinibacillus.***

46.1.2 Species definition

The nature of bacterial species has long been a subject of debate. For many years the problem was complicated by the absence of sexual reproduction in bacteria; without interbreeding populations, bacterial species lack the genetic cohesion that sexual reproduction provides. Further, bacteria lack complex morphological features, and at one time most phenotypes were characterized by data on enzyme activity, substrate utilization, or chemical constituents of the cell. Today, the characters used are predominantly molecular or genomic. In practice, taxonomic description of a bacterial species requires specification of the boundary that circumscribes it, i.e. that encloses the strains that are valid members of the species and that excludes all others.

Several different molecular or genomic methods are used to provide the data needed for the

circumscription or definition of bacterial species. The relative usefulness of 'innovative methods' of a molecular and genomic nature introduced into the field of bacterial taxonomy was examined by an 'ad hoc committee for the re-evaluation of the species definition in bacteriology', as reported by Stackebrandt *et al.* (2002). Interestingly, most of those methods had been reviewed by Wayne *et al.* (1987). Four methods are described in outline here.

1. 16S rRNA sequencing. This approach, which requires that the strains of a given species show >97% rRNA sequence identity has been used extensively. Theoretically, the sharing of >97% 16S rRNA similarity could be used as an absolute boundary for the circumscription of species but, as described below, the resolving power of 16S rRNA is insufficient to guarantee correct delineation of bacterial species. However, it contributes to knowledge of bacterial phylogenies. The replacement of 16S rRNA sequencing with high-quality rDNA sequencing has improved the determination of inter-species and intraspecies relatedness.

2. Multilocus sequence typing (MLST) enables elucidation of genomic relatedness at both intra-species and inter-species levels by sequence analysis of alleles in a minimum of five housekeeping genes that are distributed across the genome. (Housekeeping genes encode for metabolic functions and are subject to stabilizing selection.) Because MLST does not describe strain relationships phylogenetically, i.e. in terms of the level of homology among sequences, but rather as sequence types in terms of similarity of alleles, it does not lead to false conclusions that might result from recombination. Strains identical at three alleles are likely to have a recent common ancestor (Maiden *et al.*, 1998; Baldo *et al.*, 2006a).

3. DNA–DNA relatedness. To determine the DNA–DNA relatedness of two taxa, the DNAs of two organisms are mixed and denatured to give a solution of mixed, single-stranded DNA molecules. Under controlled conditions, DNA reassociation occurs resulting in hybrid molecules: the higher the genetic similarity of the two organisms, the more hybridization will occur. It is recommended that values of 70% or higher 'relative binding ratio' (RBR) are reasonable boundaries for species circumscription (Rosselló-Mora and Amann, 2001).

4. Average nucleotide identity (ANI) was proposed for defining prokaryote species and classifying groups. Cells from a strain designated as the nomenclatural type, and ideally perpetuated in living cultures, are used as a reference in DNA sequencing investigations. Comparison of the gene content of 70 closely related and fully sequenced bacterial genomes to identify species boundaries showed the ANI of the shared genes of any two strains to be a robust means for comparing genetic relatedness between them, and that ANI values of 94% corresponded to the 70% DNA–DNA association of the traditionally accepted species definition. The evolutionary distance between two strains could be measured by the average nucleotide density of all conserved genes as computed by the BLAST algorithm (Konstantinidis and Tiedje, 2005). For taxonomic purposes, the analyses need sequence only 20% of the genome of the query strains, and not their full sequences (Richter and Rosselló-Móra, 2009).

The accuracy of 16S rRNA sequencing in species identification (>97% similarity required) was compared with the accuracy of DNA relatedness (70% or more similarity required between pairs or triplets of taxa). In an experimental comparison of the two methods, the 16S rRNA measurements indicated species distinctness when RBR values did not. Thus, for the three species *Staphylococcus pisciferm entans*, *Staphylococcus carnosus* and *Staphylococcus condimenti*, the determined values were 16S rRNA measurements of 98.9–99.9%, but RBR values of only 51–58%. For the species *Natronobacterium tibetense* and *Natronobacterium bangense*, the determined values were a 16S rRNA of 95.2%, but an RBR of only 35% (Rosselló-Mora and Amann, 2001).

In a review titled 'The species concept for prokaryotes', Rosselló-Mora and Amann (2001) described the factors that render the problem so complex, discussed the importance of genomic

information and proposed a 'phylo-phenetic' concept of prokaryotic species which led to the following definition: 'a phylo-phenetic species is a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and (that) is diagnosable by a discriminative phenotypic property'.

Individual species of bacteria may occupy a number of different habitats, and not just transiently, so their exposure to different selective forces leads over time to the appearance of ecotypes; this necessarily widens the boundary that circumscribes the species (Konstantinidis and Tiedje, 2005).

46.1.3 Gram-positive and Gram-negative bacteria

Most bacterial taxa fall into one or other of two groups, Gram-positive and Gram-negative, which are structurally distinct, differ in certain biological characteristics, and can be distinguished by their response to Gram's crystal-violet stain.

Gram$^+$ bacteria. The cell wall is composed of: (i) an inner plasma membrane; and (ii) a thick multilayered peptidoglycan layer. These bacteria are highly resistant to physical disruption and drying. When exposed to Gram's stain, the cell wall peptidoglycan layer is stained a dark blue or violet. A positive response to Gram's stain is characteristic of the phylum 'Firmicutes', which includes the families Bacillaceae (including *Bacillus*, *Lysinibacillus*) and Clostridiaceae (including *Clostridium*) (Section 46.1.1). A few firmicute taxa, e.g. the class Mollicutes (including the Spiroplasmataceae, with *Spiroplasma*, lack a cell wall beyond the plasma membrane, and so do not respond to Gram staining.

Gram$^-$ bacteria. The cell wall is composed of two membranes separated by a periplasmic space: (i) an inner plasma membrane (a phospholipid bilayer); and (ii) an outer membrane composed of lipopolysaccharides with an inner phospholipid 'leaflet'. Within the periplasmic space is a single, thin peptidoglycan layer. These bacteria are only weakly resistant to physical disruption and drying. The cell wall cannot retain Gram's crystal-violet stain owing to protection by the outer layer and the small amount of peptidoglycan in the periplasmic space. A negative response to Gram's stain is characteristic of the phylum Proteobacteria, including the Rickettsiales (e.g. *Wolbachia*).

46.2 BACTERIA OF THE MOSQUITO GUT

The microbial flora of healthy insects is largely limited to the gut. In any locality, the gut flora consists of indigenous bacteria that enter with the food intake and that survive the prevailing conditions, e.g. pH, digestive enzymes and availability of oxygen and nutrients.

46.2.1 Aquatic stages

Bacteria are abundant in the sites at which mosquito larvae feed, and are a major constituent of the larval gut contents (Volume 1, Section 4.1.1). For example, bacteria were predominant among identifiable organisms in the midgut of *Ochlerotatus triseriatus* larvae collected from tree holes, and of *Anopheles quadrimaculatus* and *Coquillettidia perturbans* larvae collected from freshwater marshes (Walker *et al.*, 1988). During metamorphosis, the alimentary canal of culicids is restructured with total replacement of the midgut (Volume 1, Section 8.4.2). During the pharate pupal stage (before larval–pupal ecdysis), regenerative cells within the midgut epithelium form a pupal midgut epithelium, and by the early pupal stage in *Anopheles stephensi*, *Stegomyia aegypti* and *Cx. pipiens* the pupal midgut epithelium is sufficiently formed to secrete a peritrophic matrix around the histolysing larval midgut epithelium and its contents. Sometimes, a second such peritrophic matrix is secreted at about the time of emergence (Moncayo *et al.*, 2005). Nitrogenous and other waste products that accumulate in the midgut lumen during metamorphosis are discharged through the anus as meconium shortly after emergence. The alimentary canal of emerging adults is free of microorganisms or contains only a few. In wild fourth-instar larvae of *Oc. triseriatus* the mean bacterial count was 39,260 per midgut, but it was only 141 in adult females newly emerged from wild-caught pupae (Table 46.1) (DeMaio *et al.*, 1996).

46.2.2 Adults – field observations

Wild-caught, non-blood-fed females of *Oc. triseriatus* collected on human bait in Maryland contained in the midgut lumen an average of 386 bacteria, of 12 species; *Pseudomonas aeruginosa* and *Serratia marcescens* occurred most frequently. Newly emerged female *Psorophora columbiae* and *Cx. pipiens* reared from wild-caught pupae or larvae contained, respectively, very few or few midgut bacteria. During the first two days after females of *Oc. triseriatus* and *Ps. columbiae* had taken a blood meal, the numbers of midgut bacteria increased to tens of thousands. However, by 96 h post-feeding the numbers had declined dramatically (Table 46.1) (DeMaio *et al.*, 1996).

Among adult female *An. gambiae s.l.* and *Anopheles funestus* caught in Kenya and Mali (total $n = 2430$), 12–28% contained Gram-negative bacteria in the midgut and 8–23% contained Gram-positive bacteria. The commonest bacteria were *Pantoea agglomerans*, which is often associated with soil and plants, and *Escherichia coli* (Straif *et al.*, 1998). The midguts of 116 indoor-resting, blood-fed females of three *Anopheles* species (*An. funestus*, *An. gambiae* and *An. arabiensis*) collected in Kenya together contained 16 species of bacteria from 14 genera. Bacteria were found in 15% of the mosquitoes; few of those harboured more than one species, and only one species of bacterium was found in more than one mosquito (Lindh *et al.*, 2005).

In the light of these findings, which are representative of others that have been reported, we may conclude that the acquisition of bacteria by non-blood fed mosquitoes during the first days of adult life is not extensive and is random as to bacterial species, and that it increases greatly after blood feeding.

Table 46.1 Numbers of bacteria in the midguts of wild-caught mosquitoes of three species. (From DeMaio *et al.*, 1996.)

	Bacteria per midgut		
Stage	*Mean*	*s.e.*	n
Ochlerotatus triseriatus			
Fourth-instar larvae, wild-caught	39,260	12,970	50
Adult females from wild-caught pupae			
Newly emerged	141	99	50
Non-blood-fed females, wild-caught			
Dissected on capture	386	237	50
24 h post-blood meal	26,220	10,000	50
48 h post-blood meal	61,400	36,150	50
96 h post-blood meal	1,232	713	50
Psorophora columbiae			
Adult females from wild-caught pupae			
Newly emerged	2	1	10
24 h post-blood meal	32,600	8,670	10
48 h post blood meal	45,840	7,360	10
96 h post blood meal	253	77	10
Culex pipiens			
Fourth-instar larvae, wild-caught	36,120	8,330	50
Adult females from wild-caught pupae			
Newly emerged	32	11	55

Aquatic habitats: *Oc. triseriatus* – beech tree holes; *Ps. columbiae* – freshwater marsh; *Cx. pipiens* – stagnant pool.

46.2.3 Effects of gut bacteria on the immune system

The microbiota present in the midgut of adult female mosquitoes has long been known to affect the development of mosquito pathogens. Micks and Ferguson (1961) reported that treatment of *Cx. quinquefasciatus* with antibiotics destroyed the gut microflora, and that in females infected with *Plasmodium relictum* it led to a doubling of the number of oocysts that formed on the gut wall.

A blood bolus forms in the midgut lumen of mosquitoes shortly after blood feeding, a time when the number of commensal bacteria is increasing, but the bacteria are unable to invade healthy cells of the midgut epithelium. After an anopheline mosquito has imbibed blood infected with *Plasmodium*, the male and female gametes combine, and the zygotes formed develop to ookinetes. In *An. stephensi* infected with *P. berghei* and kept at 21°C, most zygotes had differentiated into ookinetes by 18 h post-infection (Al-Olayan *et al.*, 2002). Several hours after ingestion of a blood meal the blood bolus becomes surrounded by secreted materials, which gradually harden to form a peritrophic matrix. The peritrophic matrix is impermeable to bacteria but permeable to ookinetes, which digest it locally and then invade the midgut epithelium. Some of the bacteria present in the midgut lumen then enter the damaged epithelial cells.

In female *An. gambiae* infected with *Plasmodium berghei*, a strong immune priming response was established when bacteria came in contact with damaged epithelial cells, the immune system developing a state of enhanced immune surveillance. Immune priming resulted in a differentiation of haemocytes, both quantitative and qualitative, which persisted for the remaining lifespan of the mosquito. Exposure to bacterial infection increased the proportion of circulating granulocytes, and triggered changes in their morphology and binding properties. Circulating granulocytes mediated responses that enhanced anti-plasmodial immunity in challenged mosquitoes.

When after 7 or 14 days mosquitoes were re-challenged with a second infected bloodmeal, survival of the oocysts that had developed from ookinetes was lower than in controls. Upon such later exposure to a similar infection, mosquitoes mounted a more effective antibacterial response which also indirectly harmed malaria parasites. In other experiments, pre-exposure of *An. gambiae* to *P. falciparum* reduced oocyst density after a later challenge with the same parasite. By contrast, elimination of the gut microbiota enhanced the infection of mosquitoes by *Plasmodium*.

Rodrigues *et al.* (2010) concluded that the mosquito immune system can adapt by modulating the abundance and responsiveness of haemocyte populations. They proposed a concept of insect innate immune memory, defined as 'long-term' functional changes in the immune system that allow an insect to mount a more effective immune response upon re-encountering the same or a similar microbe.

Peptidoglycans are a class of glycoconjugates found only in bacterial cell walls, and their recognition is essential for insect antibacterial defence. In *Drosophila melanogaster*, the transmembrane PGN Recognition Protein LC (PGRP-LC) is a receptor of the Imd signalling pathway that is infected after infection with bacteria, mainly $Gram^+$. Bacterial infections in *An. gambiae* are sensed by the orthologous PGRP-LC protein, which then activates a signalling pathway that involves the Rel/NF-KB transcription factor Rel2 (Section 42.4.2). At early stages of haemolymph infections with the $Gram^+$ *Staphylococcus aureus*, PGRP-LC signalling led to transcriptional induction of antimicrobial peptides. The size of mosquito symbiotic bacteria populations and their dramatic proliferation after a bloodmeal, as well as intestinal bacterial infections, were also controlled by PGRP-LC signalling. This defensive response modulated intensities of infection of mosquitoes with *Plasmodium berghei* in the laboratory, and with *P. falciparum* field isolates. Alternative splicing of PGRP-LC transcripts produced three main isoforms, of which PGRP-LC3 appeared to have a key role in the resistance to bacteria and modulation of *Plasmodium* infections. Structural modelling indicated that PGRP-LC3 is capable of

binding monomeric peptidoglycans but unable to initiate dimerization with other isoforms (Meister *et al.*, 2009).

46.3 GROWTH AND SPORULATION

(a) Growth cycle and spore formation

Cell multiplication of bacteria is by binary fission. Under laboratory conditions of batch culture in enclosed vessels, the bacteria first adapt to the growth conditions, synthesizing RNA, enzymes and other molecules. This is the *lag phase* of the growth cycle when they are unable to divide (Table 46.2). The *exponential phase* that follows is characterized by cell doubling, when the number of new bacteria appearing per unit time at any given moment is proportional to the population density, and the logarithm of cell number increases linearly with time. Because of this geometric progression, there is a direct relationship between the number of cells initially present in the culture and the number present after a period of exponential growth, such that

$$N = N_0 2^n \qquad (46.1)$$

where N is the final cell number, N_0 is the initial cell number, and n is the number of generations that occur during the period of exponential growth. At one point during the growth of a cell, a septum forms across that cell, leading to two processes: in one part of the cell the appearance of a nascent spore, and in the other part duplication of the cell's genetic material prior to the formation of two genetically identical cells by binary fission. As the spore develops, it becomes dehydrated, protective outer coatings are deposited, and when mature it is released from the cell. Upon germination the spore contents rehydrate and a new bacterium emerges from it.

After a number of hours of batch culture, the culture medium becomes unsuitable as a result of depletion of nutrients and the accumulation of waste products to an inhibitory concentration, and a *stationary phase* starts. The growth rate slows until the reproductive and death rates equalize and there is no net change in cell number. In most bacteria, the stationary phase is followed by the *death phase*, when the cell numbers falls to zero. In reality, even in batch culture, the cells do not reproduce in synchrony without explicit prompting, and the four phases are not fully distinct.

Table 46.2 Terms used in descriptions of the growth of bacterial populations under conditions of batch culture.

Generation time. The time required for the formation of two cells from one by binary fission.
Growth. An increase in the number of cells, or of cell mass, in a population.
Growth rate. The change in cell number or cell mass per unit time.
Lag phase. The period between inoculation of bacteria into a fresh medium and the start of the exponential phase of growth during which synthesis of RNA, enzymes and other molecules occurs but the cells are unable to divide. Absent if the inoculum is taken from a culture in exponential growth.
Exponential (or logarithmic) phase. The period of population increase that is characterized by repeated cell doubling. Plotting the natural logarithm (ln x) of cell number against time produces a straight line, the slope of which depends upon the growth conditions. For most bacteria, sporulation occurs during this phase.
Stationary phase The period immediately following the exponential phase during which there is no net increase or decrease in cell number or mass because the culture conditions do not allow continued growth owing to an absence of nutrients and/or an accumulation of toxic waste products. Endospore-producing bacteria form endospores during this phase.
Death (or decline) phase. A period after which provision of nutrients has ceased and cells die.

(b) Growth cycle with formation of endospores

Bacillus, *Lysinibacillus* and *Clostridium* are among the many Firmicute genera that produce endospores. After formation of a septum across the cell, a thick internal wall encloses the developing spore's DNA and part of its cytoplasm. The primary function of the endospore is to ensure survival of the bacterium through periods of environmental stress. Endospores are dormant and resistant to desiccation, extremes of temperature, UV radiation and chemical attack, and commonly occur in soil or water, where they can survive for long periods. In species of *Bacillus* and *Clostridium*, the endospores are not formed and released during the exponential phase but during the stationary phase, when the changes that stop population growth trigger sporulation, which takes about 8 hours. The vegetative cells differentiate to sporangia, a sporangium being the bacterial cell plus a single, large spore within a membranFigureous coat, the exosporium. Seven stages have been distinguished in the process of sporulation: Stage 1, transformation of the chromatin into an axial filament within the cell; Stage II, completion of a septum towards one pole which produces two cells, the smaller being the 'prespore' (or forespore) and the larger the 'mother cell'; Stage III, engulfment of the prespore by the mother cell; Stage IV, deposition of two layers of cell wall material between the membranes that surround the engulfed spore; Stage V, deposition of layers of coat material around the prespore; Stage VI, maturation – the prespore matures into a heat-resistant spore but is still situated within the mother cell; and Stage VII, lysis of the mother cell and release of the mature spore (Piggot, 2004, review). Partial growth curves for *L. sphaericus* and *Clostridium bifermentans* in batch culture are shown in Figures 46.15 and 46.17, respectively.

At the innermost part of an endospore are its genomic material, ribosomes and cytoplasm, and large amounts of dipicolinic acid that appear to play a role in maintaining spore dormancy. Those components, which exist in a very dehydrated state, are surrounded by a unit cell membrane. Beyond that are a number of layers of different thickness. The different components of endospores are given different names by different authors. The genomic material and cytoplasm are often termed the 'core', and the cell membrane the 'inner membrane'. They are surrounded by a multilaminated layer composed of loosely cross-linked peptidoglycan (or cortex), and beyond that are a thin membrane (outer membrane), an electron-dense spore coat (coat) composed of keratin-like protein, and in some species an outermost layer, the exosporium, which is composed of lipid and proteins. The resilience of endospores can be explained largely by these surrounding structures: for example, the cortex prevents dehydration of the core, which provides resistance to high temperature; and the spore coat provides chemical and enzymatic resistance. The outer layer of peptidoglycan is hydrolysed during germination, while the inner layer of peptidoglycan is retained during germination. An endospore can remain dormant for many years, but can transform into a vegetative cell relatively rapidly (Madigan *et al.*, 2003).

(c) Endotoxins

In certain bacteria, toxins are synthesized during sporulation and remain enclosed within the sporangia. These are 'endotoxins'. In *L. sphaericus*, the endotoxins are formed as a parasporal inclusion body within the exosporium; in *B. thuringiensis*, with very few exceptions, a similar inclusion body is formed outside the exosporium, and is independent of the spore after the mother cell lyses. Retention of parasporal bodies within exosporia does not increase their stability. In distilled water at 30°C, the residual toxicities of the spores and inclusion bodies of *L. sphaericus* and *B. thuringiensis*, concentrated by centrifugation, had both declined to 30–40% after 5 months. In polluted water at 30°C, the toxicities of both fell by 60–70% within 1 week, and by about 95% within 2 weeks (Nicolas *et al.*, 1994).

The growth cycle ends with lysis of the sporangia and release of the endospores and endotoxins (Madigan *et al.*, 2003).

46.4 THE BACTERIAL GENOME

46.4.1 The genome

The genomic DNA of most bacteria is contained in a single, circular chromosome, but up to about 4% is contained in plasmids – circular, double-stranded DNA molecules that replicate independently of the chromosome, and that are naturally transferable from one individual to another by the process of conjugation.

Many of the toxin genes of certain species of the Bacillus Cereus Group, including *Bacillus anthracis* and *B. thuringiensis*, are located in large plasmids; for example, in *B. anthracis* two large plasmids, pXO1 and pXO2, code for exotoxin and capsule respectively. Certain isolates, e.g. from a tannery dump site or digested sewage sludge, showed most of the genomic characteristics of *B. anthracis* but lacked pXO2 and were avirulent (Turnbull *et al.*, 1992). Although pXO1 and pXO2 could be transferred into *B. cereus* in the laboratory, only bacilli with a *B. anthracis* genotype were found able to accommodate the plastids (Priest and Dewar, 2000). However, isolates of *B. cereus* have been found to harbour plastids very similar to pXO1 or pXO2 (Section 46.5.1.b).

Most subspecies of *B. thuringiensis* that have been investigated encode their insecticidal toxins on extrachromosomal elements. The plasmid pBtoxis of *B. thuringiensis israelensis* encodes for all of the Cry and Cyt toxins of that subspecies and for other toxins also (Section 46.5.3.c). Over 23% of the genes on pBtoxis resemble transposon-related genes, so a considerable amount of DNA exchange has occurred during its evolutionary history (Berry *et al.*, 2002). The plasmid pAW63, present in *B. t. kurstaki*, can conjugate efficiently for both its own transmission and that of small mobilizable plasmids, and it is capable of transfer to *B. t. israelensis* and *B. cereus* (Van der Auwera *et al.*, 2005).

46.4.2 Conjugation

Bacterial conjugation is a mode of unidirectional transfer of genetic information involving direct contact between a donor and a recipient cell. In this process, DNA is transferred from donor to recipient bacteria by a complex of proteins, the conjugation apparatus. To a certain degree, conjugation mimics sexual reproduction. The process is widespread among bacteria; it can occur within or between species of different genera or even of much higher taxa, and enables bacteria to rapidly acquire new traits. For conjugative transfer to occur, an intimate association between the cell surfaces of the donor and recipient cells is necessary. In Gram-negative bacteria, this physical contact is established by complex extracellular filaments termed pili. Gram-positive bacteria lack pili, and intercellular adhesion is effected by 'adhesion' or 'aggregation' substances that cover the surface of the donor cell. Close association between two cells triggers a series of steps that leads to transport of donor DNA through a conjugation pore. The DNA is transported as a nucleoprotein particle composed of a protein, relaxase, that is covalently bound to the 5' end of a molecule of ssDNA (Schaechter, 2004; Christie *et al.*, 2005).

Intraspecies and inter-species conjugation within and between Cereus Group species is described in Section 46.5.1.b. Genetic elements that may be transferred during conjugation include: (i) plasmids of two sizes, smaller and larger, which differ in biological characteristics; and (ii) conjugative transposons. Incorporation of these replicons into the recipient's chromosome allows 'chromosome mobilization', which results in a high frequency of recombination. Viral bacteriophages (Section 46.9.8.a) occur commonly in *B. thuringiensis*, and their ability to transfer plasmids between *B. thuringiensis*, *B. cereus* and *B. anthracis* has been widely used in the laboratory; however, no evidence of phage-mediated gene transfer among *B. thuringiensis* in the natural environment has been reported (Glare and O'Callaghan, 2000; Schaechter, 2004).

Type IV secretion systems (T4SSs) are one of a number of bacterial mechanisms that transport DNA or protein-DNA complexes across cell membranes from donor to receptor cells. Bacterial conjugation is a particular form of this mechanism, and a close evolutionary relationship has been shown between T4SSs and bacterial conjugation.

T4SSs also function to export toxic molecules into eukaryotic host cells. Type IV secretion systems assemble as translocation channels at the envelopes of bacteria, often with either a pilus or the protein adhesin, where they serve two purposes: (i) genetic exchange or gene acquisition; and (ii) delivery of effector molecules, including proteins and DNA-protein complexes, to prokaryotic and eukaryotic target cells. As constituents of T4SSs, ATPases of subclass VirB11 are essential for type IV secretion (Christie *et al.*, 2005).

46.4.3 Clonal assemblages

The members of some bacterial species seldom exchange DNA. In the absence of sexual reproduction they are barely genetically transformable, and develop in nature as assemblages of clones. Each clone is genetically distinct, and consists of the offspring of a single parent derived by asexual reproduction. The members of a clone may be widely distributed geographically, but can be recognized by multilocus enzyme electrophoresis.

Data obtained by multilocus sequence typing (MLST) (Section 46.1.2) can be used to determine population structures by analysing the extent of linkage disequilibrium between alleles and looking for recombination by the non-congruence of gene trees and the presence of significant mosaic structures. For highly clonal species, the phylogenetic relationships between isolates can be inferred from suitably derived dendrograms (Maiden *et al.*, 1998).

In nature, a mutation that allows a bacterium to outcompete other members of the species will spread, and in the absence of genetic exchange the genome that contains the mutant locus remains intact as that bacterium replaces its conspecifics. Non-competitive clones become extinct, and the successful clones become widely disseminated. Such purging of diversity results in a distinctive clonal population structure. In contrast, in species in which DNA exchange is frequent, e.g. genetically transformable bacteria such as *Bacillus subtilis*, it is rare to find two isolates with identical chromosomes because strains are continually subject to incoming DNA. In such populations, the chromosome is a changing mosaic of DNA sequences, and there are no opportunities for clonal populations to develop.

Populations of *L. sphaericus* are strongly clonal, and chromosomal typing reveals that strains of *B. anthracis* also constitute a homogeneous clonal population. Priest and Dewar (2000) asserted that strains of *B. thuringiensis israelensis* are a clone in which plasmid composition and toxin types are consistent wherever in the world the isolates originated. As one example they cited, nine strains isolated from soil samples from different regions of Brazil, and characterized as serotype H–14 (*B. t. israelensis*), had virtually identical whole-cell protein PAGE electropherograms (Kaji *et al.*, 1994).

46.5 *BACILLUS THURINGIENSIS*

Bacillus Cohn (1872) is a large genus of rod-shaped, endospore-forming, usually motile, Gram-positive bacteria. The species are aerobic or facultatively anaerobic, and under appropriate environmental conditions almost all produce spores that are resistant to high temperatures and desiccation. For many years the genus was unified by easily recognizable traits, e.g. rod-shaped cells that produced endospores under aerobic growth conditions. Eventually, advances in genomic and phylogenetic analysis led to the transfer of species to new genera, e.g. to *Paenibacillus* in 1994, *Brevibacillus* in 1996 and *Lysinibacillus* in 2007, which were characterized by their rRNA gene sequences.

46.5.1 Systematics

Ideas on the nature of bacterial species have developed in recent years (Section 46.1.2). Identifying the boundaries of taxa assigned to the *Bacillus cereus* species group has proved exceptionally problematic, and it is necessary to be aware of current ideas on that topic when considering the species status of *B. thuringiensis*.

(a) The Bacillus cereus group

This group comprises six species: *Bacillus anthracis*, *Bacillus cereus* and *Bacillus mycoides* which were described in the 19th century; *Bacillus thuringiensis* which was described in 1915; and *Bacillus pseudomycoides* and *Bacillus weihenstephanensis* which were described in 1998. At one time, the methods used to distinguish species were based on flagellar serotype and biochemical characteristics. In addition, isolates of *B. thuringiensis* could usually be distinguished by the presence of parasporal bodies, but some natural acrystalliferous strains were assigned to *B. thuringiensis* (Lecadet *et al.*, 1999). *Bacillus mycoides* and *B. pseudomycoides* could be distinguished from the other species by the formation on agar plates of rhizoidal (root-like) colonies, and from one another by biochemical characters (Nakamura and Jackson, 1995; Nakamura, 1998). *Bacillus weihenstephanensis* was first distinguished as a psychrotolerant species, able to grow at 7°C or below (Lechner *et al.*, 1998).

Relationships between species of the Cereus Group proved refractory to investigation by traditional taxonomic methods, but investigations that made use of a variety of DNA-based typing methods yielded consistent results. PCR analysis of repetitive patterns scattered through the genome confirmed the relatedness of the six members of the Cereus Group (Cherif *et al.*, 2003). Phylogenetic analyses that used phenetic or genomic data always showed *B. cereus*, *B. anthracis* and *B. thuringiensis* grouped together and separate from the other species (e.g. Xu and Côté, 2003), while advanced genetic techniques showed that those three species have extensive genomic similarities and few consistent differences (Priest *et al.*, 2004).

The monomorphic nature of the *B. anthracis* strains, and their similarity to *B. cereus*, suggest that *B. anthracis* is a descendant of a *B. cereus*-like ancestor, and one that gained competitive advantage by harbouring plasmids with virulence genes. Expression products of those genes cause death of the host and so enable subsequent growth in the carcass.

A multilocus analysis of housekeeping genes in strains of five Cereus Group species led to a phylogenetic tree based on 'sequence types' in

which 59 strains grouped into three clusters and nine major branches (Priest *et al.*, 2004). Where the strains were identical with strains analysed by Hill *et al.* (2004), the tree resembled one produced by those investigators (Figure 46.1). Interspersion of strains assigned to *B. cereus* and *B. thuringiensis* occurred even in branches composed of only a few lineages. The authors recommended that the nomenclature of bacteria be modified so that lineages are named by sequence-type designations in accordance with the clonal structure of the populations.

The relationships between members of the Cereus Group are non-linear and complex, probably resulting from cycles of isolation and niche expansion, and facilitated, at least in part, by horizontal gene transfer (Han *et al.*, 2006). The knowledge that the Cereus Group evolved as asexually derived clonal populations provided a unifying concept. Priest and Dewar (2000) commented that, whatever the typing procedure, clones of *B. cereus* and *B. thuringiensis* are intermingled, but rarely if ever are strains of *B. cereus* and *B. thuringiensis* included in the same clone. They suggested that the two taxa be considered a collection of intermingled clones that is diverging into species. Plasmid maintenance may be a driving force in this divergence; transfer of plasmids between bacterial cells occurs, but only certain combinations of plasmid and host can compete and survive in the environment.

In most sections of this chapter, topics may be described and discussed independently of the niceties of microtaxonomy, so for pragmatic reasons the conventional species names are used for taxa of the Cereus Group.

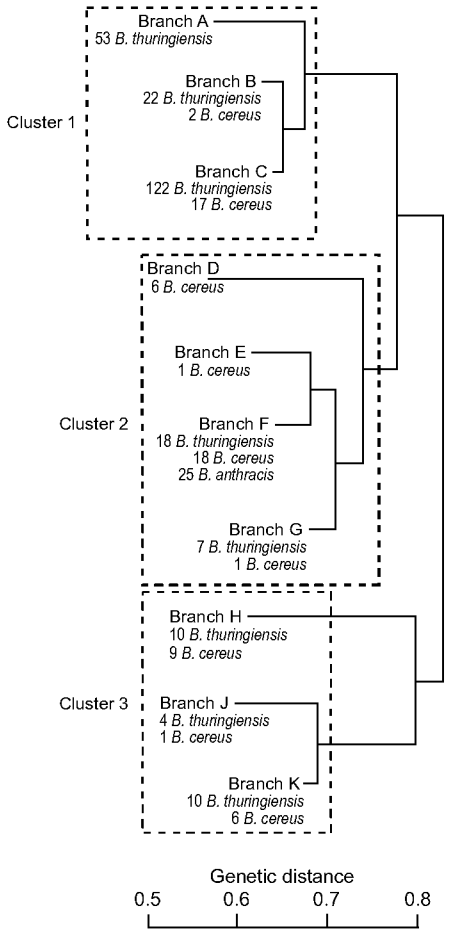


Figure 46.1 A phylogenetic tree based on AFLP typing of DNA from isolates of *Bacillus cereus*, *Bacillus anthracis* and *Bacillus thuringiensis*. (After Hill *et al.*, 2004.) Fluorescent amplified fragment-length polymorphism (AFLP) analysis was undertaken of DNA from 332 isolates (*B. cereus*, 61; *B. anthracis*, 25; *B. thuringiensis*, 246) originating in 17 or more countries. The isolates grouped into three clusters and a total of ten branches (A–H, J–K). Most branches included both *B. cereus* and *B. thuringiensis* isolates. All *B. anthracis* isolates mapped to branch F.

(b) Conjugation in the Cereus Group

As noted in Section 46.4.2, the unidirectional transfer of genetic information by conjugation between donor and recipient cells can occur within or between species, genera or much higher taxa of many bacteria, and enables them to acquire new traits.

In the laboratory, toxin-encoding plasmids can be passed from crystalliferous to acrystalliferous strains of *B. thuringiensis*, and from *B. thuringiensis* to other species. In mixed culture, plasmids were transferred from *B. thuringiensis* to *B. cereus*. Expression of the toxin-encoding genes in *B. cereus* was confirmed by the production of parasporal crystals (González *et al.*, 1982). In these Gram$^+$

bacteria, the plasmid transfer factors are ascribed to the conjugative 200 kb plasmid pXO16. Two aggregation phenotypes of *B. t. israelensis* were identified, Agr^+ and Agr^-, which produced visible aggregates when exponentially growing cells were mixed in broth. Plasmid transfer was unidirectional, from Agr^+ to Agr^- cells. Loci essential for the Agr^+ phenotype have been localized on plasmid pXO16, a plasmid-transfer system that is thought to mobilize plasmids of distinct replication types (Grohmann *et al.*, 2003, review).

In attempts to transfer the plasmid pBtoxis by conjugation from *B. t. israelensis* to 15 strains of *Bacillus cereus* group species (*B. cereus*, *B. thuringiensis kurstaki*, *B. mycoides* and *B. weihenstephanensis*), only one *B. cereus* strain and one *B. t. kurstaki* strain became transconjugants, and then at low transfer rates (Hu *et al.*, 2005). Transfer of pBtoxis from *B. t. israelensis* to *L. sphaericus* by conjugation produced transconjugants with enhanced pathogenicity to *St. aegypti* (Gammon *et al.*, 2006).

The transfer of highly mobile plasmid-encoded genes is likely to occur within the cadavers of their insect hosts on leaf surfaces. Conjugation may also occur in nutrient-rich soil or decaying organic matter. Although the chance of conjugation in those habitats is poor owing to low *B. thuringiensis* densities, it is compensated for over time by their almost universal occurrence (Glare and O'Callaghan, 2000, review).

(c) Evolution and population structure of the Cereus Group

Two investigations provided information on the evolution of the Cereus Group, one based on AFLP (amplified fragment-length polymorphism) analysis of DNA and the second involving multilocus sequence typing (MLST) to measure genomic relatedness. In the first investigation, phylogenetic trees were deduced from AFLP finger printing of the DNA in strains of Cereus Group species. Many strains were from national collections and had been identified to species; the others were from diverse sources including soil, clinical isolates and food products that had caused diarrhoea or vomiting, some being provisionally assigned to species (Hill *et al.*, 2004; Hoffmaster *et al.*, 2006). The strains grouped into ten branches (A–H and J–K), which further sorted into three clusters (Figure 46.1). In the first study, involving 332 Cereus Group strains, three of the branches contained lineages of one species only; six branches contained lineages of two species; and one (branch F) included lineages of all three species. Branch A was formed of 53 strains, all identified as *B. thuringiensis*. Not shown in Figure 46.1, the strains of each *B. thuringiensis* subspecies in branch A tended to segregate in adjacent lineages. The 61 strains that formed branch F were assigned to species as follows: *B. cereus*, 18; *B. thuringiensis*, 18; and *B. anthracis*, 25 (Hill *et al.*, 2004).

A detailed illustration of Branch F (Figure 46.2) reveals the interspersion of species. The 36 strains of *B. cereus* and *B. thuringiensis* are distributed between many interspersed small clades or subbranches, in most cases each involving just one of those two species. The strains assigned to *B. cereus* and *B. thuringiensis* show wide genetic diversity. In contrast, all 25 *B. anthracis* strains group alone in a single clade. These independently derived strains of *B. anthracis* are monomorphic, suggesting that they constitute a distinct species, despite the close genetic relationship of the clade to some strains of *B. cereus* (Hoffmaster *et al.*, 2006).

Insights into possible ways in which clones have arisen were obtained by AFLP analysis of DNA from isolates/strains of bacteria believed to have been the causal agents of pathogenesis in human or animal hosts (Table 46.3). All formed lineages in Branch F of the Cereus Group tree (Figure 46.2; see arrows). Their interspersion between lineages of *B. anthracis*, *B. cereus* and *B. thuringiensis* is consistent with episodes of conjugation having occurred. The strain *Bt* 97-27 was assigned to *B. t. konkukian*, which contains a plasmid (pAW63) that is very similar to the *B. anthracis* virulence plastid pXO2. Plasmid pAW63 can conjugate for its own transmission and for that of small mobilizable plasmids; it is capable of transfer to *B. t. israelensis* and *B. cereus* (Van der Auwera *et al.*, 2005). Other strains (*Bc* G9241, *Bc* 03BB87, *Bc* 03BB102) contained genetic sequences

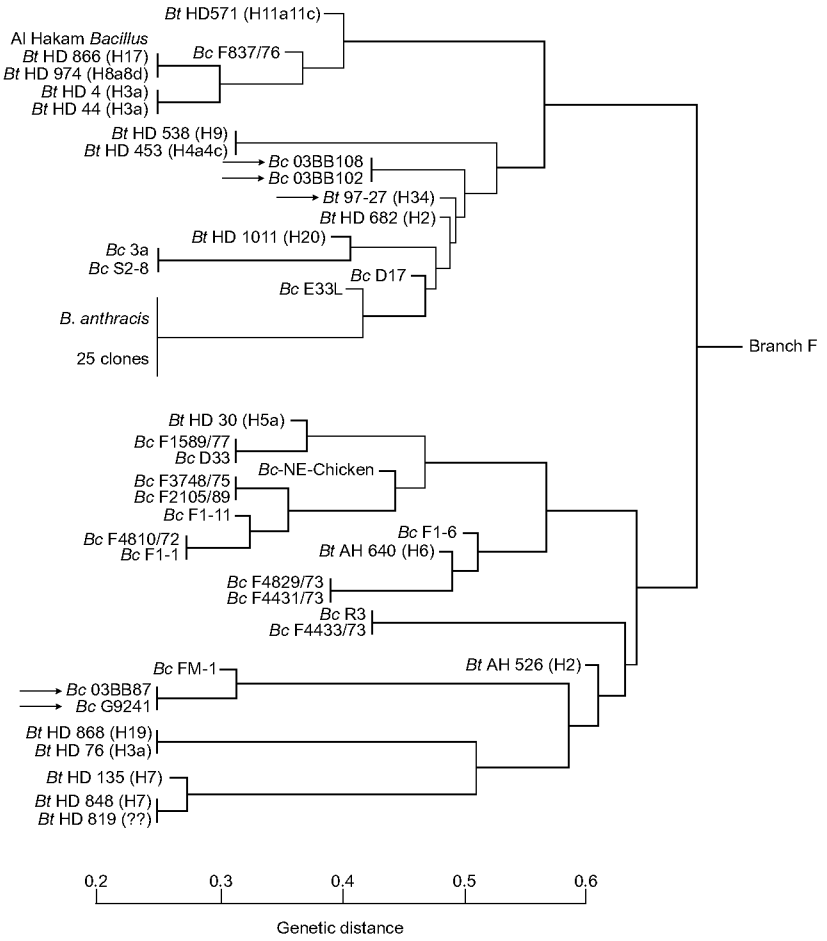


Figure 46.2 Branch F of a phylogenetic tree of *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*. (After Hoffmaster *et al.* (2006) as modified by P.J. Jackson, a co-author.) The branching of the tree corresponds to that of the tree in Figure 46.1, but the phylogeny was derived independently by AFLP (amplified fragment-length polymorphism) analysis of DNA from a partly different set of isolates and strains. Lineages assigned to *B. cereus* are indicated by the prefix *Bc* with isolate or strain number. The 25 *B. anthracis* isolates formed a single clade, and are not named. Strains of *B. thuringiensis* (prefixed *Bt*) are from the 'Howard Dulmage Collection of *Bacillus thuringiensis* strains' and indicated by the prefix HD and the strain number. For *B. thuringiensis* strains and isolates, the H antigen number (with prefix H) is also shown; this indicates the serotype and hence the subspecies to which they belong. Arrows indicate five isolates and strains of *B. thuringiensis* mentioned in the text.

Key to subspecies of *B. thuringiensis* identified by H antigen number: H2, *B. t. finitimus*; H3a, *B. t. alesti*; H4a, 4c, *B. t. kenyae*; H5a, *B. t. canadensis*; H6, *B. t. entomocidus*; H7, *B. t. aizawi*; H8a, 8b, *B. t. morrisoni*; H9, *B. t. tolworthi*; H11a, 11c, *B. t. kyushuensis*; H17, *B. t. tohokuensis*; H19, *B. t. tochigiensis*; H20, *B. t. pondicheriensis*; H34, *B. t. konkukian.*

Other isolates and strains. (i) Al Hakam *Bacillus*. From a site about 50 km from Baghdad, Iraq. Has no plasmids; shows few characteristics of *B. thuringiensis* or *B. cereus*. (ii) *Bc*-NE-Chicken. Isolated from chicken that caused food poisoning in a patient in Nebraska. (iii) *Bt* AH 526. Isolated from soil on Jeløy Island, Moss, Norway. Information from Dr Paul Jackson (co-author).

Table 46.3 Details of isolates or strains assigned to *Bacillus cereus* (*Bc*) or *B. thuringiensis* (*Bt*), which had been isolated from infected or dead hosts or their close environment.

Isolate	*Source*	*Characteristics*	*Refs*
Bt 97-27 *	Necrotic tissue in French soldier wounded by land mine; Bosnia, 1995.	Contained plastid pBT0727, which was very similar to *Bacillus anthracis* virulence plastid pXO2.	1, 2
Bc E33L	Zebra carcass in Namibia, but probably an environmental isolate and not the cause of death.	Closest lineage to *B. anthracis* clade in the dendrogram (branch F of the Cereus Group tree, Figure 46.2).	2
Bc G9241	Causative agent of severe pneumonia in welder; Louisiana, 1994.	Genome shared significant homology with *B. anthracis* genome sequences, and harboured almost the entire *B. anthracis* plasmid pXO1.	3
Bc 03BB87	Metal worker who died from pneumonia; Lubbock, Texas, 2003.	Contained pXO1 genes; indistinguishable from *Bc* G9241 isolated in Louisiana.	4
Bc 03BB102	Metal worker who died from pneumonia; San Antonio, Texas, 2003.	Contained pXO1 toxin genes and *cap A, B, C* genes for capsule biosynthesis in *B. anthracis.*	4
Bc 03BB108	Work site of worker in San Antonio.	As *Bc* 03BB102.	4

*, A strain of *B. t. konkukian*.
References: 1, Hernandez *et al.* (1998); 2, Han *et al.* (2006); 3, Hoffmaster *et al.* (2004); 4, Hoffmaster *et al.* (2006).

which, to a greater or lesser extent, resembled those of the *B. anthracis* plasmid pXO1. The area (Texas and Louisiana) in which the strains that caused severe pneumonia were collected coincided with areas where anthrax occurred naturally in herbivores (Hoffmaster *et al.*, 2006). It was not known whether the *Bacillus anthracis* toxin genes found in those strains were expressed or whether any expressed toxins played a role in virulence.

The investigators surmised that the first appearances of different groups of strains, e.g. branches A to K (Figure 46.1), were separated by many generations, and that specific phenotypes, whether pathogenic or insecticidal, had been acquired after the ancestor of each branch had appeared. The interspersions are consistent with the idea that horizontal gene transfer of plasmids or other genetic elements played a role in the evolution of phenotypes. Branches arose from ancestral strains that expanded clonally and then inherited *B. cereus*- or *B. thuringiensis*-like phenotypes by horizontal gene transfer. Over 90% of the *B. thuringiensis* strains with insecticidal attributes mapped to the three branches of cluster 1, suggesting that those branches had acquired plasmids that conferred insecticidal properties (Hill *et al.*, 2004). The tendency of strains of particular *B. thuringiensis* subspecies to cluster together supports their subspecies rank.

In a later investigation, a comprehensive evolutionary analysis of the Cereus Group was undertaken by analysing a whole database of multilocus sequence data from 667 Cereus Group strains (including 333 unique sequence types). A key question concerned the genetic structure of the virulent strains. Of the 667 strains, 31 were members of *B. anthracis* and 124 had been isolated 'from human infections or from ingredients shown to have been directly responsible for food poisoning'. The infections were grouped into six pathogenicity classes: anthrax cases, 31 strains; emetic food poisoning with vomiting, 15 strains; blood (neutropenia, endocarditis, pyrexia), 29 strains; diarrhoea, 22 strains; pneumonia and lung infections, 14 strains; and infected wounds, 44 strains. The remaining 512 strains were not associated with disease. A phylogenetic analysis showed that the strains grouped into three major clades.

Clade 1 (208 strains): comprising all *B. anthracis*, half of the *B. cereus* and a few *B. thuringiensis* strains. *B. anthracis* and all strains associated with emetic food poisoning, half of strains associated with septicaemia.
Clade 2 (370 strains): comprising half of the *B. cereus* and most *B. thuringiensis* strains; half of strains associated with septicaemia.
Clade 3 (89 strains): comprising the *B. mycoides*, *B. weihenstephanensis* and several undetermined strains.

Of the six pathogenicity classes, the strains from anthrax cases and from emetic, toxin-producing strains showed a more clonal structure, and were closely related within clade 1. The other clinical strains of *B. cereus* were evenly distributed between and within clades 1 and 2.

The patterns of genetic exchange showed partial barriers to gene flow between the three clades. The pathogenic strains did not exhibit atypically high or low rates of recombination, consistent with the members of the Cereus Group being opportunistic pathogens (Didelot *et al.*, 2009).

(d) Serotypes and subspecies of B. thuringiensis

Bacterial strains that are novel and have been characterized to a certain degree may be assigned a strain number. At one time, the category 'variety' was used for strains that were sufficiently distinct and for former species that were reduced in rank. Following the introduction of serological methods to characterize bacteria, serological variants were termed serotypes, and eventually the serotypes were ranked as subspecies.

Heimpel and Angus (1958) distinguished and keyed six Cereus Group species and their varieties. Three of those species, with a total of six varieties, had the ability to form a crystalline parasporal body in the sporangium; today, only the *B. thuringiensis* var. *thuringiensis* of these authors is still recognized (as a subspecies). de Barjac and Bonnefoi (1962) analysed 35 cultural and biochemical characteristics of 24 crystal-forming strains, and produced a key which placed them in six groups, which corresponded to the six varieties distinguished by Heimpel and Angus (1958); they also undertook a serological study of flagellar antigens (which they termed H antigens), as determined by agglutination tests in tubes, and this placed the 24 strains in the same six groups. In time, the early agglutination assays were superseded by miniaturized microplate assays (Laurent *et al.*, 1996). The differentiated groups were regarded as serotypes of *B. thuringiensis*, but the term serovariety, or more commonly serovar, was also used.

Until relatively recently, to identify an isolate, or to characterize it if novel, an antigenic suspension of the isolate was prepared and tested against reference H antisera representative of the known serotypes. Any strain not detected by any of the reference antisera was a potential new serotype. An antiserum directed against an antigenic suspension of such a strain was prepared and tested against H antigens of all known serotypes to find whether or not they were agglutinated by it. An isolate not detected by any of the reference antisera was considered a new serotype, and the antigen that characterized it was assigned a number with the prefix H. If a cross-reaction with other antigenic factors occurred, the antiserum saturation technique was used to identify possible immunological variants within a known serotype. Current genomic techniques (Section 46.1.2) will now be used.

Bacillus thuringensis serovar *thuringiensis*, characterized by possessing antigen H1, was known for its pathogenicity to caterpillars, and since the 1930s has been used as a bioinsecticide (although outperformed by *B. t.* serovar *kurstaki* (H3a3b3c)). The first mosquitocidal form to be investigated was antigenically distinct from the 13 known serotypes, so it was designated serotype H14 and named *B. thuringiensis* var. *israelensis* after its country of origin (Section 46.5.11.h) (de Barjac, 1978). Just over 20 years later, Lecadet *et al.* (1999) reported that 82 serovars had been identified among the 3500 isolates of *B. thuringiensis* in the IEBC collection (International Entomopathogenic Bacillus Collection of the Pasteur Institute), but many of the names had not been formally published. Eventually, it was recognized that the serovars of *B. thuringiensis* have the characteristics of subspecies, and they are now ranked as subspecies; for example, *B. thuringiensis* serovar *kyushuensis*

(H11a,11c) is now the subspecies *B. thuringiensis kyushuensis* (often written as *B. thuringiensis* subsp. *kyushuensis*) (Table 46.4). Glare and O'Callaghan (2000) listed 54 named subspecies of *B. thuringiensis*.

(e) Brevibacillus laterosporus

The spore-forming bacterium *B. laterosporus* produces parasporal bodies. Sporangia with spores in different stages of development were found to contain three types of parasporal body, described as lamellar, homogeneous and striated. The predominant lamellar type was composed of ~20 layers, and in its final form was canoe or keel shaped. With the spore, it almost filled the sporangium. A second type, of 100–200 nm diameter, was homogeneous in electron density. A third type was rod shaped, with a diameter of 200 nm or more, and appeared striated with alternating light and dark bands (Montaldi and Roth, 1990).

Two strains of *B. laterosporus*, LAT 006 and 16-92, the latter isolated from dead insects, were selected for mosquito larvicidal activity, and gave rise to strains designated 921 and 615, respectively, which also produced crystalline inclusions. Assays of final whole cultures or of resuspended pellet fractions of the two selected strains, when quantified in spores ml^{-1}, gave LC_{50} values against *St. aegypti* and *An. stephensi* larvae said to be equivalent to those of *B. thuringiensis israelensis*. The LC_{50} values against *Cx. pipiens* larvae were one to two orders of magnitude higher. Fractionation of sporulated cells of strain 615 yielded a purified crystal fraction composed of cube-shaped crystals. The LC_{50} for *St. aegypti* was 3.0 ng ml^{-1}, and that for *An. stephensi* 5.0 ng ml^{-1}, said to be similar to the toxicities of *B. thuringiensis israelensis* crystals (cf. Table 46.6) (Orlova *et al.*, 1998).

46.5.2 Formation of parasporal bodies and their ingestion

(a) *Parasporal body formation*

During the exponential phase, vegetative cells may produce exotoxins, secreting them into the external medium. Unlike most other species of *Bacillus*, during sporulation cells of *B. thuringiensis* produce proteinaceous, insecticidal endotoxins that are sequestered as protoxins within a crystalline inclusion. In most subspecies of *B. thuringiensis*, they appear as one or more crystalline, proteinaceous, parasporal bodies situated within the sporangium (the cell) but outside the exosporium (Figure 46.3A). After lysis of the sporangia, the spores and parasporal bodies disperse separately. However, in a very few subspecies or strains parasporal bodies may form inside the exosporium. In *B. t. finitimus*, a parasporal body appears first inside the exosporium, and during the final stage of sporulation another parasporal body appears in the usual place, outside the exosporium. Both contain a major protein of 135 kDa (Debro *et al.*, 1986). In certain strains of *B. t. oyamensis*, a crystalline parasporal body forms within the exosporium and not elsewhere (López-Meza and Ibarra, 1996).

The parasporal bodies of some subspecies of *B. thuringiensis* are bipyramidal, but those of *B. t. israelensis* are roughly spherical, averaging 0.7–1.2 μm diameter, and consist of three distinct inclusions numbered 1, 2 and 3. Each inclusion is enclosed in one or more layers of a fibrous envelope, and several layers of similar material surround the parasporal body (Figure 46.3B).

Inclusion 1. Comprises >50% of the parasporal body. It is pleiomorphic, does not stain readily with osmium tetroxide (and so is of low electron opacity), crystallizes in a lattice with a 4.3 nm repeat, is soluble at pH 9.2, lytic to mammalian haemocytes, toxic to *St. aegypti* larvae, and composed of at least 11 polypeptides, the major ones being two polypeptide doublets (molecular masses 140 and 135 kDa, and 27 and 26.5 kDa). Oligomers formed in multiples of the paired 27 and 25.5 kDa peptides might account for the peptides of higher molecular mass. The 27 kDa protein is more abundant in this inclusion than in the others.

Inclusion 2. Usually rod-shaped, it is osmiophylic (staining readily with osmium tetroxide, and so of higher electron opacity), a crystal lattice with repeat of *c.* 7.8 nm, soluble at pH

Table 46.4 The crystal proteins and toxins of some mosquitocidal subspecies of *Bacillus thuringiensis*.

Subspecies	*Flagellar antigen*	*Crystal protein name*	*Solubilized crystal protein (kDa)* *	*Toxin (kDa)* †	*Refs*
B. t. israelensis	H 14	Cry4Aa	135	20+45	1, 2, 10, 16
		Cry4Ba	128	60–68	10, 16, 17
		Cry10Aa	?58¶		26, 27
		Cry11Aa	72 ‡	32+34	10, 16, 18, 19
		Cyt1Aa	27	25	2, 10, 16
		Cyt1Ca §			27
		Cyt2Ba	24	22	24, 28
B. t. morrisoni	H 8a8b	Cry1Bc	144		10, 11, 21
		Cry4Aa	135		10, 11
		Cry4Ba	128		10, 11
		Cry11Aa	72 ‡		10, 11
		Cyt1Aa	27		10, 11
B. t. medellin	H 30	Cry11Bb	84	30, 35	6, 7
		Cry29A			13
		Cry30A			13
		Cyt1Ab	28	25	4, 12,
		Cyt2Bc	30		12
B. t. jegathesan	H 28a28c	Cry11Ba	81		15
		Cry19Aa	75		5
		(ORF2)	(60)		5
		Cry24Aa	76		21
		Cry25Aa	76		21
		Cyt2Bb	30	24	14
B. t. darmstadiensis	H 10a10b	Cry5Aa	152		21
		Cry5Ab	142		21
		Cyt2Aa	28	23	3, 20, 25
B. t. kyushuensis	H 11a11c	Cyt2Aa	58	23	8, 9, 22, 23

Equivalence of obsolete code names for crystal proteins with names in current use: CryIVA = Cry4Aa; CryIVB = Cry4Ba; CryIVD = Cry11Aa; CryVA(a) = Cry5Aa; CytA = Cyt1Aa; CytB = Cyt2Aa or Cyt2Ba according to subspecies (Crickmore *et al.*, 1998).
*, In some instances, it is not certain which of a number of precursors is the natural protoxin.
†, In some instances, different investigators reported slightly different molecular masses for the same toxin. The values cited here are those listed in the Swiss-Prot Protein Knowledgebase (www.expasy.ch/sprot/), where they are rounded to the nearest integer.
‡, Reported as 65 kDa in early publications.
§, Predicted from the plasmid gene *cyt1Ca*.
¶, In its native conformation, before exposure to denaturing agents or organic solvents, the protoxin exists as a dimer of 58 kDa (J.Li, personal communication).
References; 1, Yamagiwa *et al.* (1999); 2, Chilcott and Ellar (1988); 3, Drobniewski and Ellar (1988); 4, Escobar *et al.* (2000); 5, Rosso and Delécluse (1997); 6, Segura *et al.* (2000); 7, Thiéry *et al.* (1997); 8, Ishii and Ohba (1994); 9, Knowles *et al.* (1992); 10, Ibarra and Federici (1986a,b); 11, Gill *et al.* (1987a); 12, Juárez-Pérez *et al.* (2002); 13, Juárez-Pérez *et al.* (2003); 14, Cheong and Gill (1997); 15, Delécluse *et al.* (1995); 16, Federici *et al.* (1990); 17, Boonserm *et al.* (2005); 18, Yamagiwa *et al.* (2002); 19, Yamagiwa *et al.* (2004); 20, Promdonkoy *et al.* (2003); 21, Crickmore *et al.* (2006); 22, Koni and Ellar (1993); 23, Li *et al.* (1996b); 24, Guerchicoff *et al.* (1997); 25, Drobniewski and Ellar (1989); 26, Thorne *et al.* (1986); 27, Berry *et al.* (2002); 28, Nisnevitch *et al.* (2006).

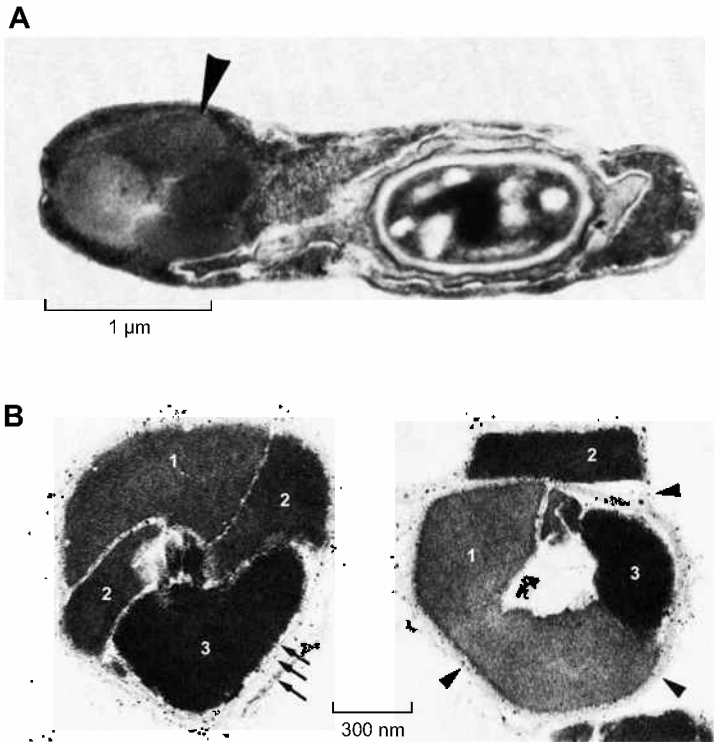


Figure 46.3 **A.** Longitudinal section through a sporangium of *Bacillus thuringiensis israelensis* from a cadaver of *Stegomyia aegypti* 24 h after infection. (From Aly *et al.*, 1985.) **B.** Representative sections through parasporal bodies of *B. t. israelensis* after purification by centrifugation. (From Ibarra and Federici, 1986b.) The numerals indicate the different inclusion types, based on differences in electron density and, to some extent, shape. The arrows point to different layers of the envelope that surrounds the parasporal body and each inclusion. The large arrowhead indicates a parasporal body. Small arrowheads indicate the envelope, where largely intact.

10.5, non-lytic to erythrocytes, of low toxicity to *St. aegypti* larvae, and contains predominantly a protein of 70 kDa which on proteolysis yields polypeptides of 35 to 40 kDa.

Inclusion 3. This is the smallest inclusion of the parasporal body and is strongly osmiophylic (of highest electron opacity) (Insell and Fitz-James, 1985; Federici *et al.*, 1990).

(b) Ingestion of parasporal bodies

Mosquito larvae that come across microorganisms or particles of organic matter are stimulated to feed. Larvae of *Aedimorphus*, *Stegomyia*, *Culex* and *Anopheles* species that fed on particles in suspension in a water column ingested spores of *B. thuringiensis* if present. Bottom-feeding larvae of *Aedimorphus vexans* aggregated where sedimented food particles were found, feeding on them and ingesting any spores of *B. thuringiensis* that were among them (Aly, 1983, 1988). Spores of *B. t. israelensis* would germinate after exposure to pH 10 buffer solution (Bhattacharya, 1999). They also germinated within the midgut of *St. aegypti* larvae, where the bacteria multiplied vegetatively and produced spores and parasporal particles. In certain feeding experiments with *St. aegypti* larvae, at 2 h post-exposure the larvae contained on average c. 10^4 spores, but by 4 h, when most larvae were dead, the number had fallen to $2–3 \times 10^2$. At 24 h the cadavers contained few spores but an increased number of vegetative cells. Subsequently, the average spore content rose steadily, reaching almost 10^5 per cadaver after 72 h (Figure 46.4). Clearly, *B. t. israelensis* could use

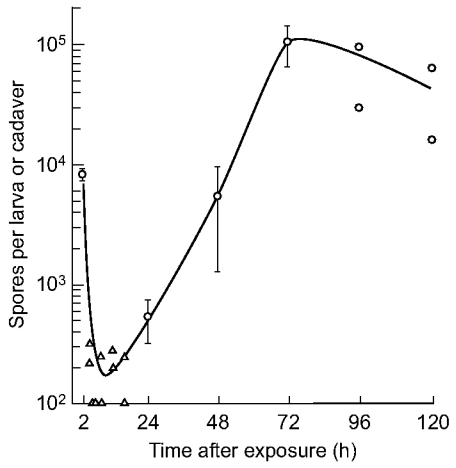


Figure 46.4 Numbers of spores of *Bacillus thuringiensis israelensis* in *Stegomyia aegypti* larvae and larval cadavers at different times after exposure to 1.9×10^4 spores ml^{-1}. Means ± s.d. The data points between 4 and 17 h are from a separate experiment. (From Aly *et al.*, 1985.)

substrates available in the cadavers for growth and sporulation (Aly, 1985; Aly *et al.*, 1985).

46.5.3 Toxins

(a) Types of bacterial toxin

The toxins produced by living organisms have very wide ranges of molecular structure, mode of action, potency and immunogenicity. Most toxins produced by microorganisms fall into one or other of two groups – exotoxins and endotoxins. Exotoxins are secreted across the cell membrane and into the surrounding medium, and those secreted by microbial pathogens can enter host cells with destructive effects. Some are enzymes, such as lecithinases, proteinases and chitinases. Certain exotoxins, e.g. botulinum toxin and pertussis toxin, have a highly specific mode of action and are exceptionally poisonous. In contrast to exotoxins, endotoxins remain associated with the microorganism until cell death and lysis. They are relatively heat stable.

Traditionally, the toxins produced by a bacterial species were distinguished by a Greek-letter prefix coupled with the word -exotoxin or -endotoxin, designated sequentially as they were discovered. Heimpel (1967) adopted this system for the four known or suspected toxins of *Bacillus thuringiensis*, which he named α-, β- and γ-exotoxin and δ-endotoxin. The γ-exotoxin, which he described as 'unidentified enzyme(s) responsible for clearing egg yolk agar', was later shown to be non-pathogenic, so the name γ-exotoxin was unfounded (Krieg and Lysenko, 1979).

The toxins produced by *B. thuringiensis* have been intensively investigated. The most important are its β-exotoxin and its δ-endotoxins. The β-exotoxin is an adenine nucleotide analogue, which is toxic to some insects when ingested and toxic to vertebrates. The δ-endotoxins are proteins that are stored within the bacterium in crystalline form until cell lysis, and that are toxic to the larvae of some insects when ingested. Strains of *Lysinibacillus sphaericus* and *Clostridium bifermentans* that are pathogenic to mosquito larvae also produce crystalline bodies composed of endotoxins.

The destruction of host cells by pathogenic bacteria involves delivery of specialized macromolecules, either into the nearby environment or by cell-to-cell transfer into target cells. Exotoxins are secreted across the microbial cell wall by the general secretion pathway (type II). Many pathogenic bacteria possess 'type IV secretion systems' (Section 46.4.2), by means of which they transfer macromolecules directly to a variety of prokaryotic and eukaryotic cells.

(b) Exotoxins of Bacillus thuringiensis

The different subspecies of *Bacillus thuringiensis* secrete a variety of substances into the culture medium; some of these are toxic to particular groups of organisms and others are not. In insects infected by *B. thuringiensis*, any exotoxins would be released into the gut lumen.

α-Exotoxin is a proteinaceous thermolabile substance, produced by certain subspecies of *B. thuringiensis*, which is toxic to some insects when ingested, e.g. larvae of *Plutella maculipennis*. Studies of α-exotoxin were stimulated by discovery of a

thermolabile 'mouse factor' produced at the end of the logarithmic phase of the growth cycle in cultures of *B. cereus* and *B. thuringiensis*. Mouse factor, or α-exotoxin, was shown not to be a phospholipase, which Heimpel (1967) had suggested. Inoculation of α-exotoxin into larvae of *Galleria mellonella* caused the destruction of haemocytes and a fatal septicaemia (Krieg, 1971a,b; Krieg and Lysenko, 1979).

β-Exotoxin, or thuringiensin, is secreted into the culture medium during the vegetative growth phase by certain subspecies of *B. thuringiensis* but not by others; it occurs in at least two forms, types I and II. It is secreted as β-exotoxin I by *B. t. darmstadiensis* and as β-exotoxin II by *B. t. morrisoni*, for both of which mosquito larvae are the most susceptible hosts (Levinson *et al.*, 1990). It is a heat-stable, dialysable compound which has a broad spectrum of activity against insects and some other arthropods. It is more toxic when administered parenterally than when ingested, but insects that have ingested high doses usually stop feeding and die within several days of exposure (Glare and O'Callaghan, 2000, review). It was toxic to larvae of *St. aegypti*, *Cx. pipiens* and *An. stephensi* at 100 g ml^{-1}, and to the adults of those species when ingested at 1 mg ml^{-1}. Sublethal concentrations delayed larval moulting (Larget-Thiéry *et al.*, 1984). Exposure of *Culex sitiens* larvae to β-exotoxin I at 0.5 or 1 mg ml^{-1} resulted in damage to columnar cells of the midgut, with deformation and shrinkage of the microvilli, and degradation of cytoplasmic organelles (Weiser and Žižka, 1994). Because of its toxicity to vertebrates, most commercial preparations of *B. thuringiensis* are prepared from isolates that do not produce this toxin. As already noted, β-exotoxin is an adenine nucleotide analogue, and it is thought to inhibit DNA-directed RNA polymerase (an enzyme that uses ATP to synthesize RNA from a DNA template) by competing with ATP for the ATP-specific enzyme binding site (Šebesta and Horská, 1970; Šebesta and Sternbach, 1970; Horská *et al.*, 1976).

Vegetative insecticidal proteins (Vips). These proteins are expressed during the exponential phase of the growth cycle, starting at mid-phase and continuing into the period of sporulation; they are secreted into the culture medium. The first vegetative insecticidal protein to be discovered, Vip3Aa, was toxic to larvae of a number of lepidopteran species. The gene *vip3A* expresses an 88.5 kDa protein which is translocated across the cell membrane and secreted into the surrounding medium (Estruch *et al.*, 1996). Ingestion of Vip3A by susceptible lepidopteran larvae caused gut paralysis at 4 ng cm^{-2} of food surface, and larval death occurred at doses above 40 ng cm^{-2}. Damage to cells of the midgut epithelium showed that tissue to be the target in susceptible species, and the consequences of cell lysis to be the cause of death (Yu *et al.*, 1997). Screening Vip3Aa1 primers against 24 subspecies of *B. thuringiensis* showed the presence of *vip*-like gene sequences in eight of them, at least seven of which included dipteran larvae among their hosts (Bhalla *et al.*, 2005). The system of δ-endotoxin nomenclature has also been applied to Vip and Vip-related proteins (Crickmore *et al.*, 2006).

Enzymes. Some 54% of *B. thuringiensis* H serotypes (n = 70) secreted chitinase into the external medium, in variable quantities. A strain of serotype H4 which was the most active in this regard, producing 355 units ml^{-1}; it was highly toxic to larvae of *Spodoptera exigua* (Lepidoptera) (Liu *et al.*, 2002). *B. t. israelensis* secreted chitinase when grown in a medium containing chitin. Exposure to a chitinase inhibitor (100 μM allosamidin) raised by a factor of 1.3 the LD_{50} of a *B. t. israelensis* preparation against larvae of the midge *Culicoides nubeculosus* (Sampson and Gooday, 1998). A chitinase gene (*ichi*) has been identified in *B. t. israelensis* (Zhong *et al.*, 2003). Phospholipase C is secreted by some subspecies of *B. thuringiensis* but not by others (Krieg, 1971a,b).

(c) *Endotoxins of* Bacillus thuringiensis

The δ-endotoxins are also known as 'insecticidal crystal proteins' (ICPs) or 'pesticidal crystal proteins'. Those produced by *B. thuringiensis* are toxic to certain insect larvae upon ingestion, and

because of their potential for control of pest insects they have been investigated in detail. δ-Endotoxins are synthesized after completion of the exponential phase of the growth cycle, during sporulation, and are assembled in one or more crystalline parasporal bodies in amounts reaching 30% of the total cell protein. The crystal proteins are insoluble under neutral conditions, but, under the strongly alkaline, and in some instances reducing, conditions found in the midguts of some insect larvae, they become soluble. The solubilized proteins are mostly protoxins, but solubilization of some innocuous proteins has also been reported. The protoxins are subjected to partial proteolytic cleavage by digestive enzymes, yielding toxins of lower molecular mass. These processes are described in greater detail in Section 46.5.6.

Between them, the subspecies of *B. thuringiensis* produce many different *δ*-endotoxins, their specificity determined by slight differences in molecular structure. Originally all names had the prefix Cry, for crystal protein, but they are now classified and named according to their amino acid sequences. Later, *δ*-endotoxins with cytolytic activity were discovered, and were given names with the prefix Cyt. These two sets of proteins are encoded by genes of two superfamilies, *cry* and *cyt*, respectively. All subspecies of *B. thuringiensis* produce Cry *δ*-endotoxins, whereas Cyt *δ*-endotoxins are produced by the few subspecies whose toxins are active against Diptera. When an individual crystal protein is formally named, the prefix Cry or Cyt is coupled with a hierarchical sequence of numbers and letters, e.g. Cry11Ba or Cyt2Bb (two of the toxins produced by *B. t. jegathesan*). The ranking and nomenclature of a number of Cry proteins are illustrated in Figure 46.5.

As noted below, Cry and Cyt proteins differ in amino acid sequence homology and in the properties of their phenotypes. Cry proteins can be defined broadly as 'found in a crystal and having toxic activity', and Cyt proteins as additionally having 'a non-specific cytolytic activity' (N. Crickmore, personal communication).

Cry proteins. These are relatively large proteins; the toxins having a molecular mass of 60–70 kDa. In many cases, but not all, the molecule is composed of three domains. Some 145 Cry *δ*-endotoxin holotypes of tertiary rank produced by *B. thuringiensis* were listed by Crickmore *et al.* (2006). In the few Cry proteins that have been investigated experimentally, the toxins bound to specific receptors on the apical plasma membrane of host midgut epithelial cells, and inserted to form transmembrane pores. That was thought to permit unregulated ion flow, leading to cytolysis and irreversible damage to the midgut epithelium. Cry toxins formed pores in cultured cells, but only in those derived from insects that were susceptible to the specific toxins.

Cyt proteins. These proteins are smaller, the molecular mass of the toxins usually falling within the range 22–25 kDa. Cyt proteins show no amino acid sequence homology to members of the Cry family. Only nine Cyt *δ*-endotoxin holotypes of tertiary rank have been described, almost without exception from subspecies of *B. thuringiensis* that are 'selectively' pathogenic to Diptera. Each toxin molecule consists of a single 'domain'. Cyt proteins interact with plasma membranes by binding to certain phospholipid components, not to protein receptors. Under *in vivo* conditions their toxic actions are specific to dipteran larvae. Under *in vitro* conditions, Cyt toxins are cytolytic to cells from Diptera, and at high concentrations to most eukaryotic cells tested (Li *et al.*, 1996b; Ellar, 1997; Guerchicoff *et al.*, 2001; Crickmore *et al.*, 2006). Whether individual Cyt proteins cause cytolysis by pore formation or by disrupting cell membranes in the manner of detergents is still under investigation (Section 46.5.9).

Dissolution of parasporal crystals from *B. t. israelensis* yields four major proteins (protoxins) with molecular masses of 135, 128, 72 and 27 kDa, which are named Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa, respectively. Proteolysis of the protoxins yields toxins of lower molecular mass (Table 46.4), all with substantial activity. Toxins produced by dissolution and proteolysis *in vitro* may vary in molecular mass and toxicity from those produced *in vivo* because the extent of cleavage at the carboxy and amino termini varies

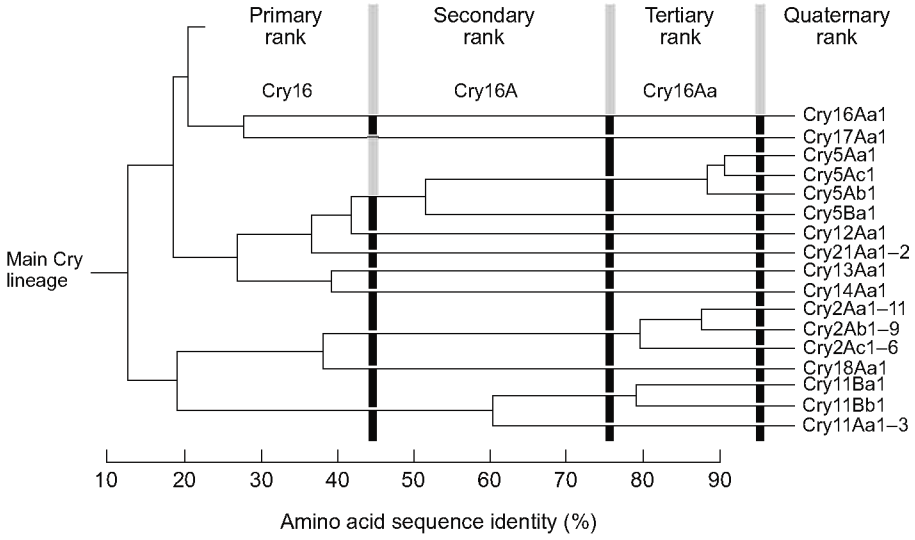


Figure 46.5 Part of a phylogenetic tree of Cry δ-endotoxins that was constructed from comparisons of full-length amino acid sequences. (After Crickmore *et al.*, 1998.) It is shown to illustrate the way in which Cry proteins are named. (The nomenclature of Cyt endotoxins has the same basis.) Quoting the authors: 'Vertical lines drawn through the tree show the boundaries used to define the various nomenclatural ranks. The name given to any particular toxin depends on the location of the node where the toxin enters the tree relative to these boundaries. A new toxin that enters the tree to the left of the leftmost boundary will be assigned a new primary rank (an Arabic number). A toxin that enters the tree between the left and central boundaries will be assigned a new secondary rank (an uppercase letter). It will have the same primary rank as the other toxins within that cluster. A toxin that enters the tree between the central and right boundaries will be assigned a new tertiary rank (a lowercase letter). Finally, a toxin that joins the tree to the right of the rightmost boundary will be assigned a new quaternary rank (another Arabic number)'. Toxins of tertiary rank are 'holotype toxins'. Lineages that are not known to have evolved beyond the tertiary rank are assigned a terminal 1, e.g. Cry16A1. In general, where lineages have not evolved beyond the tertiary rank, use of the two terminal indicators is optional and dictated only by the need for clarity. Thus, Cry16Aa1 can be referred to as Cry16A. Nodes that fall to the right of the rightmost vertical line are not drawn on this tree. The leftmost vertical line indicates *c.* 75% homology; the central line indicates *c.* 45% homology; and the rightmost indicates *c.* 95% homology. These percentages are not fixed but result from partly subjective decisions on where the boundaries of rank should lie. They can be changed by the *B. thuringiensis* Pesticidal Crystal Protein Nomenclature Committee. (N. Crickmore, personal communication.)

with the conditions. Cyt1Aa showed greatest activity when the protoxin had been processed at both termini to the more active 22 kDa form (Al-yahyaee and Ellar, 1995). In addition to those toxins, Cry10A and Cyt2B are expressed at very low levels, while the gene *cyt1Ca* predicts a protein of unusual structure.

As noted earlier, the insecticidal toxins of *B. thuringiensis* are encoded on extrachromosomal elements. In a number of mosquitocidal subspecies, all genes necessary for synthesis of the δ-endotoxins are contained in a single, large plasmid. In the case of *B. t. israelensis*, the plasmid is named pBtoxis, has a genome of 128 kbp, and has been completely sequenced. Among the many proteins expressed by pBtoxis genes are the seven δ-endotoxins listed in Table 46.4. Of these, four (Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa) are expressed at high levels and two (Cry10Aa, Cyt2Ba) at very low levels. The predicted product of the pBtoxis gene *cyt1Ca* is a 'possible two-domain toxin', with an N-terminal half similar to Cyt1Ab of *B. t. medellin*, and a C-terminal half that resembles toxins with a ricin-B lectin domain (Berry *et al.*, 2002). Messenger RNA was detected in 29 of 40 coding sequences in pBtoxis. A transcriptional survey of those genes, using known orthologues in other organisms, revealed toxin-related sequences

and genes that might have direct effects on the host phenotype, including sequences associated with sporulation, germination, cell division, enzyme production and transcriptional regulation (Stein *et al.*, 2006).

Individual protoxins can be produced by cloning single genes on replicative plasmids and expressing them in non-crystal forming strains of *B. thuringiensis* or in other bacteria. All of the endotoxin genes of *B. t. israelensis* have been cloned individually and sequenced. The proteins Cry4A and Cry4B, which are of approximately 130 kDa, are similar in structure to insecticidal crystal proteins of *B. thuringiensis* subspecies with lepidopteran activity. The 72 kDa protein Cry11A has significant homologies with Cry2Aa. The 27 kDa protein Cyt1A, which has haemolytic activity, is structurally quite different from the other mosquitocidal proteins; it constitutes 40% of the total crystal protein (Boyle and Dean, 1990; Ben-Dov *et al.*, 1996). In contrast with those proteins just named, the concentration of Cyt2Ba in the crystals is very low (Nisnevitch *et al.*, 2006).

46.5.4 Relative toxicities of the δ-endotoxins

Because a highly alkaline midgut is necessary for the dissolution of crystal proteins, the range of insect taxa that are susceptible is limited. Other characteristics of susceptible hosts are the secretion of proteolytic enzymes that cleave the protoxins at specific sites, so producing activated toxins, and the presence on the apical plasma membranes of midgut cells of appropriate receptor proteins or phospholipids. The Cry proteins of the various subspecies of *B. thuringiensis* are sometimes described as specific to larvae of a single insect order, usually to larvae of the Lepidoptera, Diptera, Coleoptera or Hymenoptera. The summarized data assembled in Table 46.5 show that the toxins of individual subspecies can affect insects of more than one order, so any specificities should be described as partial. Many δ-endotoxins have been tested on only a very few potential hosts.

Studies of the δ-endotoxins produced by different subspecies of *B. thuringiensis* have included measurement of the death rates induced by exposure to the crystals, the protoxins and the toxins. Estimates of the toxicity of the same entity by different investigators can differ by 10- to 100-fold owing to differences in assay protocol, e.g. larval age, manner of exposure to the toxin, etc. Further, cultivated strains of a subspecies obtained from different laboratories can differ markedly in potency (Brownbridge and Onyango, 1992). When exposed to suspensions of toxin particles, larval density affected the death rates of *St. aegypti* and *An. quadrimaculatus* larvae but not those of *Cx. quinquefasciatus* larvae (Aly *et al.*, 1988). Despite these limitations, the following characteristics have been established: (i) the native parasporal crystals of different subspecies can differ substantially in toxicity to mosquito larvae; (ii) the toxins from any parasporal crystal differ from one another in toxicity and cytolytic activity; and (iii) mosquito species differ in their sensitivity to the crystals and toxins from individual subspecies.

Research on the mosquitocidal subspecies of *B. thuringiensis* has focused principally on the subspecies *israelensis*, and less intensively on the five other subspecies. A report of their relative toxicities to three mosquito species is summarized in Table 46.6. The two most pathogenic subspecies were *B. t. israelensis* and *B. t. morrisoni*, the parasporal bodies of which contain four protoxins in common (Table 46.4). The parasporal bodies of *B. t. morrisoni* contain an additional protein, Cry1Bc1, of about 144 kDa, which is said to promote colloid osmotic lysis by binding to insect midgut epithelial cells of unknown function (Ibarra and Federici, 1986a; Gill *et al.*, 1987a; Crickmore *et al.*, 2006).

Bacillus thuringiensis subspecies *medellin* and *jegathesan* are somewhat less pathogenic than *B. t. israelensis* and *B. t. morrisoni*. *Bacillus t. jegathesan* was first isolated in Malaysia. Its parasporal crystals contain seven major proteins with molecular masses of 16–81 kDa. Five of these proteins and one ORF (open reading frame) are listed in Table 46.4. Immunoblotting showed that the Cry11B protein of *B. t. jegathesan*, which accounted for most of the toxicity of the native crystals, contained many amino acid sequences similar to those of Cry11A of *B. t. israelensis*. The protein Cry19A of *B. t. jegathesan*

Table 46.5 Susceptibility and non-susceptibility of larvae of four insect orders to eight subspecies of *Bacillus thuringiensis*. (From the data of Glare and O'Callaghan, 2000.) In the review from which the data were taken, mosquito larvicidal activity was reported in 32 of 54 subspecies of *B. thuringiensis*. This curtailed table is presented to provide a general impression of the susceptibility and non-susceptibility of larvae of four insect orders to eight subspecies of *B. thuringiensis* that are referred to elsewhere in Chapter 46. The data have two important limitations: (i) the numbers of species are a measure only of the numbers of species tested, and reflect degree of interest; (ii) the records of susceptibility and non-susceptibility are presented, as in the review from which they were taken, without discriminating measurements of toxicity, so they indicate only tendencies to susceptibility or non-susceptibility.

Subspecies of B. thuringiensis	*Diptera*		*Lepidoptera*		*Coleoptera*		*Hymenoptera*	
	*Genera**	*Species*	*Genera**	*Species*	*Genera**	*Species*	*Genera**	*Species*
B. t. thuringiensis								
Susceptible	20	31	190	266	20	25	9	11
Non-susceptible	10	17	20	22	26	27	8	9
B. t. israelensis								
Susceptible	47	103	5	5	–	–	8	9
Non-susceptible	18	21	12	12	21	27	1	1
B. t. morrisoni								
Susceptible	7	13	11	12	–	–	–	–
Non-susceptible	4	9	–	–	–	–	–	–
B. t. medellin								
Susceptible	3	5	–	–	–	–	–	–
B. t. jegathesan								
Susceptible	4	8	–	–	–	–	–	–
B. t. darmstadiensis								
Susceptible	9	14	10	11	2	2	–	–
Non-susceptible	5	8	7	7	2	2	–	–
B. t. kyushuensis								
Susceptible	4	7	3	3	–	–	–	–
Non-susceptible	4	5	4	4	–	–	–	–
B. t. kurstaki								
Susceptible	19	33	234	330	14	15	22	25
Non-susceptible	24	28	41	55	28	35	37	65

*, Genera with one or more species that are susceptible or non-susceptible.
For orders of insects other than the four entered here, the total numbers of susceptible genera and species were: *B. t. thuringiensis*, 22 and 28; *B. t. israelensis*, 4 and 4; *B. t. kurstaki*, 29 and 34; *B. t.* subspecies *morrisoni*, *medellin*, *jegathesan*, *darmstadiensis* and *kyushuensis* combined, 4 and 4. *Bacillus thuringiensis kurstaki* is included in the table as a subspecies extensively used as a bioinsecticide. Eight mosquito species were named as susceptible to it.

is a holotype toxin. The only significant similarities between its amino acid sequences and those of other *B. thuringiensis* toxins are in five blocks that are conserved in the amino-terminal region of most δ-endotoxins. The ORF2 sequence of *B. t. jegathesan* closely resembles the carboxy-terminal region of the 135 kDa toxin of *B. t. israelensis*. Tested alone, Cry19Aa1 had very low toxicity, but when assayed together with the product of ORF2 the toxicity increased more than 50-fold, with an LC_{50} of 187 ng ml^{-1} (Delécluse *et al.*, 1995; Kawalek *et al.*, 1995; Seleena *et al.*, 1995; Cheong and Gill, 1997; Rosso and Delécluse, 1997).

Purified parasporal crystals from *B. t. kyushuensis* caused only very low death rates when ingested by larvae of three mosquito species; solubilized protein

Table 46.6 The toxicities of purified parasporal crystals from six mosquitocidal subspecies of *Bacillus thuringiensis* to the larvae of three mosquito species when assayed under identical conditions. Measurements of the haemolytic activities of solubilized crystal proteins are also given. (After Ragni *et al.*, 1996.)

Subspecies of B. t. thuringiensis	*Toxicity* (LC_{50})			*Haemolytic activity* (HD_{50})
	St. aegypti	Cx. pipiens	An. stephensi	
B. t. israelensis	20	20	38	0.7
B. t. morrisoni	26	21	51	1.1
B. t. medellin	158	77	51	7.7
B. t. jegathesan	240	77	165	49
B. t. darmstadiensis *	1,103	207	538	278
B. t. kyushuensis *	55,760	13,162	12,333	85

LC_{50} values are expressed as ng ml^{-1} of purified crystals. They were the means of three assays (except where indicated) against fourth-instar larvae of *Stegomyia aegypti* and *Culex pipiens* and third-instar larvae of *Anopheles stephensi*. The LC_{50} values determined in this series of assays were substantially higher than those of some other investigators.
Haemolytic dose (HD) is expressed as mg solubilized crystal protein in 1.5 ml suspension of sheep erythrocytes.
*, Results of a single assay.

exhibited moderate haemolytic activity (Table 46.6) (Ragni *et al.*, 1996). Among the solubilized and protease-treated crystal proteins of this subspecies, only the 23 kDa component Cyt2Aa showed mosquitocidal activity, with an LC_{50} of 51.4 μg ml^{-1} against third-instar larvae of *St. aegypti* (Ishii and Ohba, 1994).

As measured by LC_{50} values against *St. aegypti* larvae, Cyt1A of *B. t. israelensis* is less toxic than Cry4B or Cry11A (Table 46.7). However, in a plot of toxicity against concentration, the slope of the Cyt1A regression line was considerably steeper than those for the Cry toxins, showing that at high concentrations it was the most active toxin (Figure 46.6). Immunological cross-reactivity can be shown between the low molecular mass toxins (of Cyt1A, Cry4B and Cry11a), which are agents of the haemolytic activity (Thomas and Ellar, 1983a; Höfte and Whiteley, 1989; Li *et al.*, 1996b; Orduz *et al.*, 1996). The three other mosquitocidal subspecies considered (*B. t. jegathesan*, *B. t. darmstadiensis* and *B. t. kyushuensis*) exhibit medium to low haemolytic activity and toxicity.

Characteristically, the native parasporal bodies are more toxic per unit weight than any of their constituent proteins, as can be seen for *B. thuringiensis israelensis* in Table 46.7, and for that reason the possibility of synergism between individual toxins has been investigated. In one investigation, expression systems were used to cause an acrystalliferous mutant of *B. thuringiensis* to produce crystals of each of the four δ-endotoxins of *B. t. israelensis*. The relative toxicities of these proteins to *St. aegypti* larvae were determined, as was that of the native crystal, and further bioassays were undertaken with mixtures of the toxins (Table 46.7). Cyt1A was the least toxic constituent. Disruption of the *B. t. israelensis* gene *cyt1A* abolished the haemolytic activity of the soluble crystal proteins but did not change their activity against larvae of *Cx. pipiens* or *St. aegypti* (Delécluse *et al.*, 1991). However, when Cyt1A was combined with any one of the Cry proteins, the activity was greater than expected from a simple additive effect. The enhancement was inversely proportional to the relative toxicity of the Cry component; thus it was three times greater than additive for Cry11A + Cyt1A, and 15 times greater for Cry4A + Cyt1A. Combinations of two or three Cry proteins also showed greater than additive effects (Crickmore *et al.*, 1995). Analysis of the results of earlier investigations showed 4-fold synergism for the mixture Cry11A and Cyt1A (0.75:1.0 w/w) and 10-fold synergism for the mixture Cry4A and Cyt1A

Table 46.7 Synergism between the δ-endotoxins of *Bacillus thuringiensis israelensis* when assayed against *Stegomyia aegypti* larvae. (After Crickmore *et al.*, 1995.)

Toxins	LC_{50}	*Expected* LC_{50}	*Positive synergism*
Native crystal	10	–	–
Cry4A	1125	–	–
Cry4B	467	–	–
Cry11A	224	–	–
Cyt1A	1209	–	–
Cry4A + Cyt1A	75	1166	15.5×
Cry4B + Cyt1A	62	674	10.9×
Cry11A + Cyt1A	118	378	3.2×
Cry4B + Cry11A	173	303	1.7×
Cry4A + Cry4B + Cry11A	125	400	3.2×
Cry4A + Cry4B + Cyt1A	77	778	10.1×
Cry4A + Cry4B + Cry11A + Cyt1A	85	481	5.6×

Transformants were produced by electroporation of an acrystalliferous mutant of *B. thuringiensis* subsp. *israelensis* in the presence of subcloned fragments of plasmid. Selected transformants were grown in culture and inclusions produced during the sporulation phase were purified. For the bioassay, 72-h-old larvae were exposed for 24 h to suspensions of purified inclusions in small volumes of water. For the toxin mixtures, toxins were combined in equal amounts by weight.

LC_{50} values are expressed in ng ml^{-1}. 'Expected LC_{50}' values for mixtures were calculated from a formula which can be extended or shortened for any number of components. The LC_{50} for a three-component mixture $LC_{50\,(m)}$ would be estimated as

$$LC_{50(m)} = \left(\frac{r_A}{LC_{50(A)}} + \frac{r_B}{LC_{50(B)}} + \frac{r_C}{LC_{50(C)}} \right)^{-1}$$

where r_A, r_B and r_C are the proportions by weight of toxins A, B and C, and a simple additive effect is assumed (Tabashnik, 1992).

(0.25:1.0; w/w) (Chilcott and Ellar, 1988; Tabashnik, 1992).

In an investigation into the role of individual toxin proteins, a strain of Cx. *quinquefasciatus* was selected for resistance against *B. t. israelensis*, while strains of *B. t. israelsensis* were produced that lacked either the *cry11A* or the *cyt1A* gene. The *Bacillus* strain lacking *cyt1A* was substantially less toxic to the mosquito larvae than the strain lacking *cry11A*, which led Wirth *et al.* (2003) to conclude that the Cyt1A toxin suppresses resistance, and in so doing synergizes the Cry proteins.

46.5.5 Alkalization of midgut fluids

When ingested by susceptible insects, the crystalline, proteinaceous parasporal bodies of *Bacillus thuringiensis* become solubilized in the alkaline environment of the host's midgut lumen, before being processed into active toxins. Mosquito larvae are among the relatively few dipterans in which the contents of the midgut are strongly alkaline.

(a) Midgut structure and ultrastructure

The midgut of a mosquito larva is a long, largely tubular epithelium surrounded by muscle fibres. At its anterior end, a short length of the midgut epithelium, the cardia, surrounds a short invagination of the oesophagus into the midgut lumen. Just posterior to the cardia, the epithelium is formed into eight sac-like gastric caeca. The remainder of the midgut consists of an anterior stomach, a short transitional region and a posterior

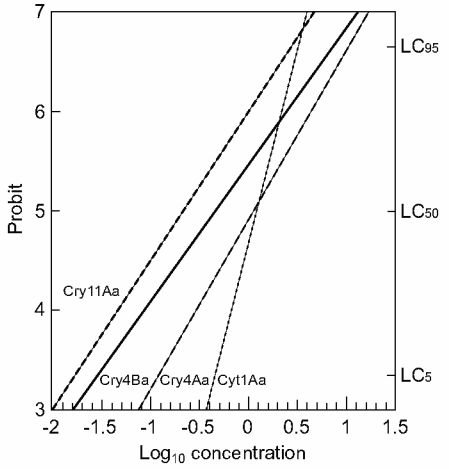
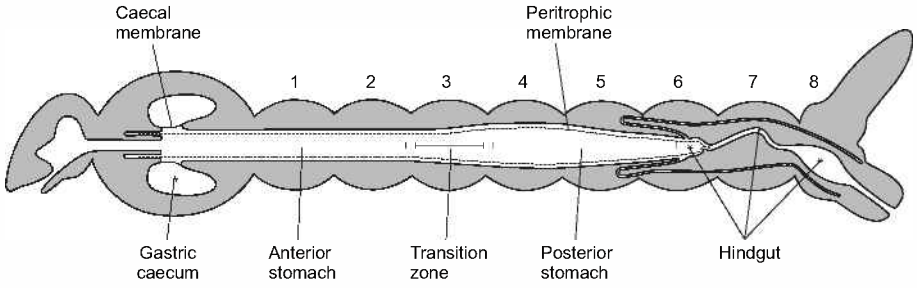


Figure 46.6 Relative toxicities to *Stegomyia aegypti* larvae of four of the δ-endotoxins produced by *Bacillus thuringiensis israelensis*. (After Crickmore *et al.*, 1995.) Regression lines of probit mortalities of the larvae against the $\log_{10}$ concentrations of the toxins. Totals of 30–60 larvae aged 72 h were exposed to four to eight concentrations of each toxin. LC_5, LC_{50} and $LC9_5$, lethal concentrations to 5%, 50% and 95% of test larvae. For further details see Table 46.7.

stomach (Figure 46.7). In *St. aegypti* larvae, those three regions form 53%, 14% and 33% of midgut length, respectively (Clark *et al.*, 2005). A cylindrical peritrophic matrix separates the midgut epithelium from the food column. The peritrophic matrices of fourth-instar larvae are multilayered and about 1 μm thick. Those of *St. aegypti* larvae are permeable to digestive enzymes and the products of digestion, but are impermeable to bacteria (Volume 1, Section 5.1.4). The lumen of each gastric caecum is separated from the tubular midgut lumen by a thin caecal membrane, which is amorphous and about 20% as thick as the peritrophic matrix. The gastric caeca are thought to be involved in ion and water transport. The gastric caeca and the stomach receive direct nervous input from two serotonergic neurons in the ingluvial ganglion.

The midgut epithelium of mosquito larvae is composed of a variety of cell types. Large columnar cells are the predominant type, accompanied by smaller cuboidal cells. Certain much smaller cells



Species						Refs
Cx. pipiens	8.0–8.5	7.5 → >10.1	→ 9.5	9.0 → 7.0	6.5–7.5	1
St. aegypti	8.0–8.5	7.5 → >10.1	→ 9.5	9.0 → 7.0	6.5–7.5	1
St. scutellaris	7.0	→ 10.0	→ 8.0	7.5 → 6.5		2
Gc. atropalpus	7.0	→ 10.0	→ 7.5	7.0 → 6.0		2
Gc. epactius	7.0	→ 10.0	→ 7.5	7.0 → 6.5		2

Figure 46.7 Regions of the alimentary canal of a mosquito larva and the pH ranges measured within their lumens for *Culex pipiens*, *Stegomyia aegypti*, *Stegomyia scutellaris*, *Georgecraigius atropalpus* and *Georgecraigius epactius*. (After Clements, 1992.) References: 1, Dadd (1975); 2, Stiles and Paschke (1980). The arrows indicate a change in pH in the direction of the arrow over the length of the gut region signified above the arrow; where numbers are present only to the right of the arrows a pH gradient up or down from the gut region to the left is indicated. The dashes indicate a range only.

are present in relatively small numbers, i.e. the regenerative, imaginal and endocrine cells. The plasma membranes of the columnar and cuboidal cells control ion fluxes. The apical plasma membrane, which faces the midgut lumen, may be extended into short or long microvilli. In some cases, the long microvilli contain narrow, elongate mitochondria. In the gastric caeca, microvilli on the apical plasma membrane of the epithelial cells contain long, slender mitochondria. The walls of the microvilli may be studded with small bodies (10–14 nm) termed portasomes. Over the basal and basolateral regions of the cells, the plasma membrane repeatedly extends inwards forming many convoluted infoldings, which may penetrate deep into the cell and often are closely associated with mitochondria. Together they form the basal labyrinth (Volume 1, Section 6.6). In cells of the anterior stomach, mitochondria are closely associated with the infoldings of the basolateral plasma membrane. In both the gastric caeca and anterior stomach, only c. 20 nm separates the surface of the mitochondria from the portasome-studded membranes (Volkmann and Peters, 1989; Zhuang *et al.*, 1999; Corena *et al.*, 2002; Clark *et al.*, 2005).

The descriptions that follow are of columnar and cuboidal cells from the gastric caeca of *St. aegypti*, *Cx. pipiens* or *An. stephensi* larvae, and of the three stomach regions of *St. aegypti* larvae.

Gastric caeca. *Columnar cells* – so-called resorptive or secretory cells. Microvilli long and thin; without mitochondria; no portasomes; presence of H^+ V-ATPase (vacuolar *H*+-ATPase) uncertain; basal labyrinth well developed; extensive rough ER (endoplasmic reticulum). *Cuboidal cells* – so-called ion-transporting cells. Microvilli long and thick, each containing a mitochondrion; plasma membrane studded with portasomes; basal labyrinth well developed; with both H^+ V-ATPase and carbonic anhydrase.

Anterior stomach. *Columnar cells* – Microvilli very short (c. 0.4 μm) or absent; infoldings of basal plasma membrane extend almost halfway into the cells, and closely associated with mitochondria and studded with portasomes; with H^+ V-ATPase. *Cuboidal cells* – Without microvilli; no carbonic anhydrase.

Transitional region. At the anterior end of this region the cells resemble those of the anterior stomach but have significantly longer microvilli; more posteriorly there is a continuous change in cell structure towards that of the posterior stomach, and the microvilli increase in length from 1.2 to 4.2 μm.

Posterior stomach. *Columnar cells* – The apical surfaces of the cells are domed and surrounded by clefts in which there are many microvilli of c. 3 μm length studded with portasomes; mitochondria are not seen within the microvilli but are abundant in both the apical and basal regions of the cells; basal labyrinth less developed than in anterior region; with both H^+ V-ATPase *Cuboidal cells* – With carbonic anhydrase.

(b) pH of the midgut lumen

The target of *B. thuringiensis* δ-endotoxins is the epithelial cells of the host midgut, and dissolution of the crystalline protoxins by exposure to high pH is an essential first step in their conversion to toxins. Many lepidopteran larvae are sensitive to δ-endotoxins, and there is a small but significant correlation between the degree of midgut alkalinity and the presence of tannins in the foliage that the caterpillars consume. Among species that fed on host plants that lacked tannins the mean gut pH was 8.25, whereas among species that fed on tannin-containing leaves it was 8.76 ($p = 0.01$) ($n = 60$) (Berenbaum, 1980). Tannin-protein complexes are relatively stable at acidic and neutral pHs, but at pH >9 most protein-tannin complexes are extensively dissociated (Van Sumere *et al.*, 1975). A high gut pH is found in some Coleoptera and Hymenoptera. Within the Diptera, this characteristic is found in aquatic larvae that feed on particulate or masticated organic matter (detritivores), including: mosquito larvae (Culicidae); blackfly larvae (Simuliidae) (Lacey and Federici, 1979; Undeen, 1979); tipulid larvae (Tipulidae) (Martin *et al.*, 1980); and, judging by their

sensitivity to δ-endotoxins, chironomid larvae (Chironomidae) (Kondo *et al.*, 1995; Karch and Delage, 2005).

By the use of pH-sensitive dyes, the pH of the midgut contents has been measured in larvae of a number of culicid species. The pH varied between different midgut regions, notably between the fluid in the gastric caeca and regions of the food column in the anterior stomach and posterior stomach (Figure 46.7). The contents of the gastric caeca are neutral in some species and weakly alkaline in others. Within the anterior stomach, the pH rises with distance posteriorly, from approximately neutral to 10. If the length of the stomach of fourth-instar *St. aegypti* larvae is c. 5 mm, of which the anterior stomach region occupies about one-half, and the pH range over the length of the anterior stomach is 7.5 to >10.1, then the mean gradient in the anterior stomach is about 1 pH unit mm^{-1}. Within the posterior stomach, the pH falls with distance posteriorly to become neutral or weakly acid. If mosquito larvae are chilled to immobility, the pH of the anterior stomach slowly falls to near neutral. Within a few minutes of return to room temperature the pH starts to rise, provided the respiratory spiracles are open at the water surface. Narcotization with CO_2 or chloroform has similar effects (Dadd, 1976).

Incubation of purified parasporal crystals of *B. t. kyushuensis* in 50 mM K_2CO_3, pH 10.5, solubilized 25% of the crystal protein, and the addition of 10 mM dithiothreitol to the buffer resulted in solubilization of over 90% of the crystal protein (Knowles *et al.*, 1992). Whether solubilization of crystal proteins ingested by mosquito larvae is increased by reducing conditions within the midgut lumen is not known.

(c) *Involvement of vacuolar H^+-ATPases*

Vacuolar H^+-ATPases (V-ATPases) are transporters with a proton-pumping action that are present on or within cell membranes and the membranes of many subcellular organelles in eukaryotic organisms. V-ATPase is a multi-subunit enzyme consisting of a membrane-bound sector, which translocates protons, and a cytosolic catalytic sector, which, by hydrolysing ATP, supplies energy for the translocation of protons. When active, these enzymes separate protons from gegenions (simple ions produced by the dissociation of a colloidal electrolyte), so producing an electrical potential difference ($\Delta\Psi$) across the membrane in which they reside. In turn, the $\Delta\Psi$ can drive other ion movements, leading directly to acidification or alkalization, and secondarily to salt transport and water movement. V-ATPases are inhibited by nanomolar concentrations of bafilomycin (Finbow and Harrison, 1997; Grabe *et al.*, 2000).

Histochemical evidence indicated the presence of V-ATPase in cells of a number of organs, including the midgut, in larvae of *Cx. quinquefasciatus* and *St. aegypti* (Filippova *et al.*, 1998). Other studies showed it to be located in the plasma membrane of certain cells, and associated with portasome particles. Volkmann and Peters (1989) described microvilli studded with 14 nm portasomes on the apical plasma membranes of ion-transporting cells in the gastric caeca of *Stegomyia*, *Culex* and *Anopheles* larvae. In *St. aegypti* larvae, antibody to the subunit E of V-ATPase co-localized with portasomes in epithelial cells in different regions of the midgut. In cells of the gastric caeca and posterior stomach, the antibody-binding sites and the portasomes were situated in the apical plasma membrane (Figure 46.8). In cells of the anterior stomach they were located in the basal plasma membrane (Zhuang *et al.*, 1999).

(d) *Ion fluxes*

The mechanisms that produce alkalization in the anterior stomach are only partly understood. In theory, active accumulation of HCO^{3-} in the lumen could account for alkalization up to about pH 8.3, sufficient for the gastric caeca and posterior stomach. To account for alkalization to values above pH 10 in the anterior stomach, additional transfer of acid equivalents (H^+) must be invoked.

Measurements of transepithelial potentials, ion concentrations and ion fluxes in preparations of *St. aegypti* larval midgut have provided pointers to

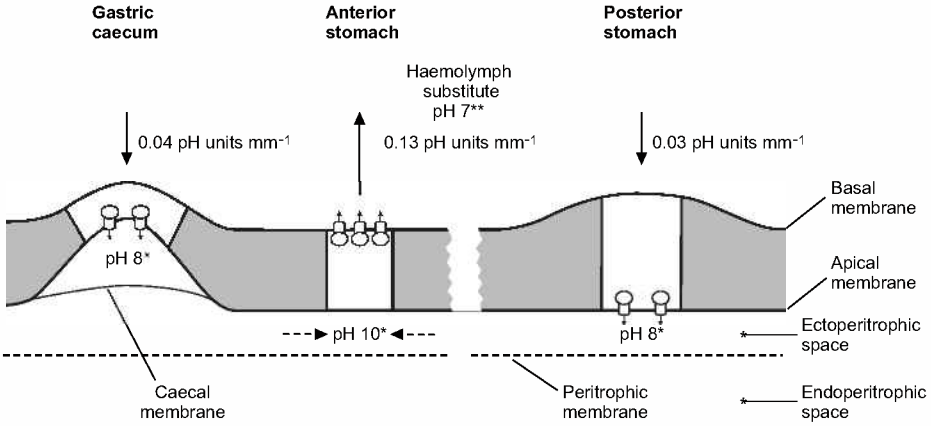


Figure 46.8 Stationary pH values and biologically generated pH gradients in bodies of fluid adjacent to the midgut epithelium of aedine mosquito larvae. (After Boudko *et al.*, 2001a.) In the semi-intact preparations used, a larva was secured and the midgut revealed through a longitudinal slit in the integument. The integument was pulled apart on either side to expose the alimentary canal with minimal damage to the tracheae and neuronal plexuses. The organs were bathed in a haemolymph substitute solution. Microelectrodes filled with a column of liquid ion exchanger were used to measure proton gradients, scanning the basal surface of the midgut at distances of 10 µm from the tissue, and then of 1–10 µm. The solid arrows indicate $[H^+]$ gradients measured with the pH-sensitive probes. The dashed arrows indicate $[H^+]$ gradients in the ectoperitrophic space measured with pH-sensitive dyes. The single asterisks indicate that the pH values were approximate. The double asterisk indicates a buffered pH value in the haemolymph substitute solution. The locations of V-ATPase molecules are shown by the symbols in the inner and outer (basal and apical) cell membranes. The same findings were obtained with larvae of *Ochlerotatus canadensis* and *Stegomyia aegypti*.

possible mechanisms. In one series of experiments, a midgut was removed, cannulated at either end, bathed in an appropriate saline, and perfused by applying negative pressure at one end. Measurements of voltage showed that the anterior stomach generates a lumen-negative, transepithelial voltage (V_{te}) of –60 mV. This decayed during the first 10–15 min after dissection, but was restored to 50% of its former magnitude when serotonin (5-hydroxytryptamine) was applied at concentrations known to be present in haemolymph. The posterior stomach has a transepithelial voltage of opposite polarity, such that V_{te} = +76 mV (Clark *et al.*, 1999, 2000; Onken *et al.*, 2004a).

Semi-intact preparations were used in investigations of the possible role of V-ATPase in alkalization of midgut fluids in larvae of *St. aegypti* and *Ochlerotatus canadensis* (Figure 46.8, see caption) (Boudko *et al.*, 2001a). Ion-selective, pH-sensitive microelectrodes were placed near epithelial cells to detect and measure pH gradients, indicative of $[H^+]$, that emanated from the apical or basal plasma membranes. Large outward $[H^+]$ gradients extended into the haemolymph from cells of the anterior stomach, with the highest concentrations close to the basal cell membrane (Figure 46.8). The gradients were steepest in the anterior part of the first abdominal segment. Much shallower, inward pH gradients were found near cells of the gastric caeca, where $[H^+]$ was lowest close to the basal plasma membrane. Small inward pH gradients were found also near cells at the middle of the posterior stomach, where they extended from the basal plasma membrane. All gradients decreased exponentially with distance from the tissue and were almost undetectable at 500 µm. Addition of the V-ATPase inhibitor bafilomycin a_1 to the haemolymph substitute greatly diminished the $[H^+]$ gradients adjacent to the anterior stomach, but had no effect on the gradients adjacent to the gastric caeca or posterior stomach. These results

were consistent with the assumption that V_{te} reflects HCO_3^- secretion to the lumen and H^+ transfer to the haemolymph.

Boudko *et al.* (2001a) commented that the extruded H^+ could be replaced by H^+ from metabolism, or alternatively by H^+ from the midgut lumen, in exchange for cell K^+. Addition of the 'strong' K^+ ion to the gut lumen and removal of the 'weak' H^+ ion would contribute to the high lumen pH, assuming that the gegenion is carbonate. Qualitatively, their results were consistent with a K^+/nH^+ antiporter on the apical membrane of the anterior stomach cells.

Also used for investigations of ion fluxes were semi-open stomach preparations, in which the anterior stomach of *St. aegypti* larvae was cannulated at one end while the other end was left open to the bath. Ion-substitution experiments showed that, for maintenance of V_{te}, Na^+ ions were required on the haemolymph side of the epithelium but not on the luminal side. V_{te} was abolished when Cl^- ions were absent from both sides of the epithelium. V_{te} was also inhibited on exposure to dinitrophenol – reversibly if the application was brief, irreversibly if prolonged. Dinitrophenol is an uncoupling agent that reduces mitochondrial ATP generation, but it can also diminish transmembrane H^+ gradients. The reversible effect of brief exposure suggested the presence of a basolateral proton pump of importance for the generation of V_{te} (Onken *et al.*, 2004a). When added at low concentration to the bathing fluid, certain peptides known to be present in *St. aegypti*, i.e. proctolin, allostatins A1–5 and neuropeptide F, reduced V_{te} in the anterior stomach. This suggested that they are involved in the coordination of ion transport (Onken *et al.*, 2004b).

In further investigations by Boudko *et al.* (2001b) with *St. aegypti* larvae, fluxes of chloride and bicarbonate/carbonate ions were, once again, measured in 'semi-intact preparations'. The chloride concentration in anterior stomach tissue was significantly higher than that in the haemolymph (p = 0.0001), while an extremely low chloride concentration was detected in fluid from the midgut lumen (luminal fluid) (Table 46.8A). In contrast, the bicarbonate/carbonate concentration was highest in the luminal fluid, significantly lower in anterior stomach tissue, and very low in the haemolymph (p = 0.0003).

Table 46.8 (A) Anion concentrations in anterior stomach tissue and adjacent body fluids, and (B) regional specificity of transepithelial chloride and acid fluxes, in the midgut of *Stegomyia aegypti* larvae. Means ± s.e. (From the data of Boudko *et al.*, 2001b.)

A. Anion concentrations

Fluid source	Cl^- *(mM)*	CO_3^{-2}/HCO_3^- *(mM)*
Haemolymph	57.6 ± 5.7	4.0 ± 2.9
Anterior stomach (tissue)	68.3 ± 5.9	50.7 ± 4.1
Luminal fluid	3.5 ± 2.6	58.1 ± 4.8

B. Chloride and acid fluxes*

Organ	Cl^- *flux* ($\mu M\ m^{-2}\ s^{-1}$)	H^+ *flux* ($nM\ m^{-2}\ s^{-1}$)
Anterior stomach	+ 32.6 ± 1.3	+ 64.6 ± 9.2
Gastric caeca	+ 17.3 ± 10.9	– 18.4 ± 9.6
Posterior stomach	+ 9.5 ± 7.6	– 20.0 ± 4.8

*, +, Efflux; –, Influx.
Anion concentrations were measured by capillary zone electrophoresis. Similar concentrations of anions were recorded in fluid from the ectoperitrophic space and the endoperitrophic space; the term luminal fluid embraces both. Fluxes of H^+ and Cl^- were calculated from $[H^+]$ and $[Cl^-]$ gradients around particular regions of the gut measured with ion-selective microelectrodes filled with a column of liquid ion exchanger.

In these further studies with semi-intact preparations of *St. aegypti* larvae, the pH gradient profiles around the midgut were the same as those shown in Figure 46.8. A spatial profile was obtained of transepithelial acid/base fluxes as an H^+ equivalent. The largest transepithelial Cl^- and H^+ effluxes were in the anterior stomach region, and corresponded to the region of highest luminal pH (Table 46.8B). In the gastric caeca and posterior stomach there was efflux of Cl^- and influx of H^+. No cationic fluxes related to alkalization could be identified, but in the anterior stomach Cl^- effluxes accompanied by acid secretion were found. The finding of strong acid and chloride effluxes from the basal surface of the anterior stomach was consistent with

the highest luminal pH being in the anterior stomach; it also provided direct confirmation of involvement of an anionic pathway in anterior stomach alkalization, and indicated that Cl^- is the principal strong-anion contribution to the net acid efflux in the anterior stomach (Boudko *et al.*, 2001b).

(e) Role of carbonic anhydrase

In aqueous solution, carbon dioxide interacts spontaneously and reversibly with water, becoming hydrated. This results in the formation of carbonic acid, some molecules of which lose a proton and become bicarbonate ions. In animal tissues, these reactions are accelerated by carbonate dehydratase, a cellular enzyme commonly called carbonic anhydrase (CA) (see Figure 46.9A). At least seven forms of the enzyme are known, CA-I to CA-VII. In mammalian tissues, bicarbonate is the blood's major buffer, and the protons produced by these reactions contribute to the lowering of pH. In mosquito larvae, bicarbonate ions are secreted into the midgut lumen where, if the pH is high, they dissociate into carbonate ions and protons. Corena *et al.* (2002) stated that a system capable of generating a high pH, such as that in the larval midgut lumen examined here, is likely to be buffered by carbonate (CO_3^{2-}), which has a pK_a of approximately 10.3. Luminal accumulation of bicarbonate might raise the pH to about 8.3, but removal of H^+ by absorption would be needed to attain pH values >10. There is some evidence that that occurs (see Subsection 46.5.5.d above).

Carbonic anhydrase was shown by a variety of techniques (histochemistry, *in situ* hybridization, ^{18}O isotope exchange) to be present in cells of the midgut epithelium of mosquito larvae, but its distribution in different parts of the midgut varied with species. For example, in *St. aegypti* and *Culex nigripalpus* it occurred at highest titre in the gastric caeca, at somewhat lower titre in the posterior stomach, and was absent from the anterior stomach. In contrast, in *An. albimanus* and *An. quadrimaculatus* it occurred at highest titre in the anterior stomach, at a lower titre in the gastric caeca, and was absent from the posterior stomach. In *Stegomyia albopicta*, CA was present in all three midgut regions. Further tests on *St. aegypti* larvae showed that CA occurs predominantly in the cuboidal cells of the midgut epithelium, and only at very low titre in the columnar cells; its distribution extended to Malpighian tubule cells and to some muscle fibres of the midgut (Corena *et al.*, 2002, 2004).

Cloning of a DNA sequence from *St. aegypti* permitted comparison with *Drosophila melanogaster* carbonic anhydrase gene sequences, and suggested that the mosquito CA isoform was soluble and had a putative molecular mass of 32.7 kDa. The gene may be expressed throughout the gut, but at highest levels in the gastric caeca and posterior stomach. Analysis of expression products revealed products of higher molecular mass in the anterior stomach and Malpighian tubules, which may represent additional CA isoforms. The analysis also showed that *St. aegypti* larvae contain more than one CA isoform. A cDNA clone revealed sequence homology to α-carbonic anhydrases from vertebrates (Corena *et al.*, 2002). Later it was found to resemble the mammalian CA-IV isoform, a GPI (glycosylphosphatidylinositol)-linked peripheral-membrane protein (Seron *et al.*, 2004).

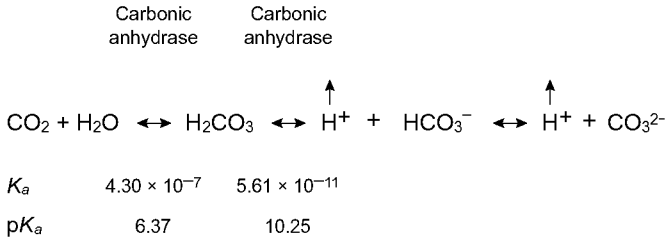
Exposure to CA inhibitors, e.g. methazolamide at 10^{-6} mol l^{-1}, blocked alkalization of the midgut contents. When the pH-sensitive dye bromothymol blue was added to the water in which larvae swam, the contents of the anterior stomach became blue and those of the posterior stomach yellow. The further addition of methazolamide caused the anterior stomach contents to become yellow, indicating a change from alkaline to acidic (Corena *et al.*, 2004).

(f) Na^+/K^+-ATPase

Na^+/K^+-ATPase (EC 3.6.1.37), also termed Na^+/K^+-exchanging ATPase or the sodium pump, is an integral, cell-membrane protein that catalyses the hydrolysis of ATP to ADP + orthophosphate. For every two K^+ ions that enter cells it pumps out c. three Na^+ ions for every hydrolysis of one ATP. Ouabain is a specific inhibitor (Smith *et al.*, 1997). By histochemical tests, Na^+/K^+-ATPase was

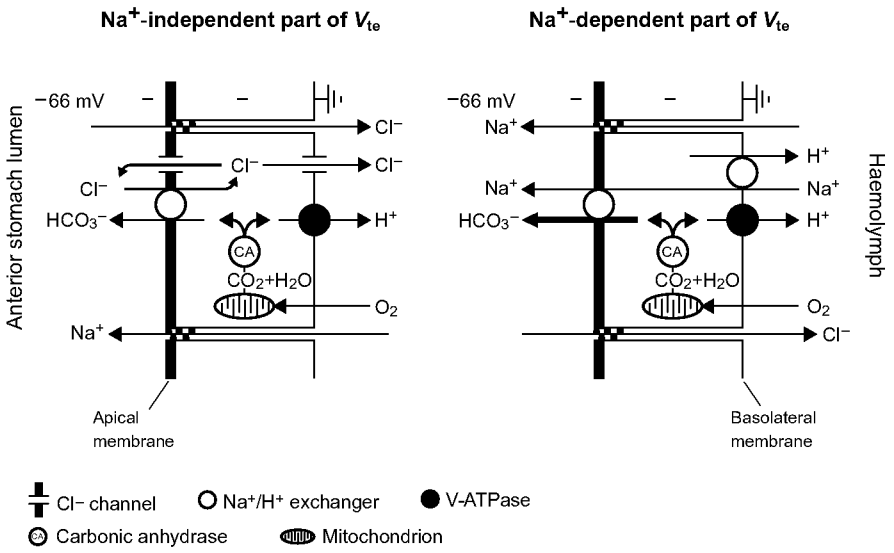
Figure 46.9 Postulated mechanism of the alkalization of the contents of the anterior stomach in *Stegomyia aegypti* larvae.

A. Formation of carbonate.



In aqueous solution, molecules of carbon dioxide are spontaneously and reversibly hydrated with the formation of carbonic acid, some molecules of which dissociate into proton and bicarbonate ions. Within animal cells, those two steps, the hydration of carbon dioxide and the formation of bicarbonate ions, are catalysed by carbonic anhydrase (= carbonate dehydratase, EC 4.2.1.1). Being a weak acid (its dissociation constant is far less than 1.0), the carbonic acid does not dissociate fully. Under conditions of high pH, some bicarbonate ions dissociate, yielding protons and carbonate ions. The symbol for the (thermodynamic) equilibrium constant of a chemical reaction is K. K_a is the symbol for the (thermodynamic) equilibrium constant for the dissociation of an acid (syn. acid dissociation constant). pK_a is the symbol for the $\log_{10}$ of the reciprocal of K_a. The values of K_a and pK_a for steps 1 and 2 in the formation and dissociation of carbonic acid, when measured at 25 °C, are cited from the *CRC Handbook of Chemistry and Physics*, 55th edn (1974).

B. Model of the alkalization of the contents of the anterior stomach in *St. aegypti* larvae, as observed in semi-open, perfused, serotonin-stimulated preparations. (After Onken *et al.*, 2004a.)



The physiological situation is reflected in the outside-negative transepithelial voltage (V_{te}). The model is based on two premises: (i) that bicarbonate ions are secreted into the stomach lumen where, if the pH is high, they will spontaneously change to CO_3^{2-}; and (ii) that acid equivalents (H^+) generated within the epithelial cells are secreted into the haemolymph.

Left. Na^+-independent part of V_{te}. Metabolic CO_2 is hydrated to form carbonic acid which then, catalysed by carbonic anhydrase, dissociates into H^+ and HCO_3^-. Protons are pumped by V-ATPase (vacuolar Na^+/K^+-ATPase) across the basolateral membrane into the haemolymph, resulting in a high cellular [HCO_3^-] and hyperpolarization of the cellular electrical potential. Cl^-/HCO_3^- exchange across the apical membrane is driven by the high cellular [HCO_3^-]. Postulated anion channels in the apical membrane allow Cl^- recycling and/or electrogenic secretion of HCO_3^-, driven by the cellular negativity. Cl^- channels in the basolateral membrane allow transcellular passage of Cl^-.

Right. Na^+-dependent part of V_{te}. It is proposed that the V-ATPase energizes transapical $NaHCO_3$ secretion via electrogenic $Na^+/2$–$3HCO_3^-$ symporters. It is thought that Na^+/H^+ exchangers in the basolateral membrane supply the cells with Na^+ and support the V-ATPases in driving H^+ across the basolateral membrane. It is proposed that transcellular secretion of Na^+ and/or absorption of Cl^-, driven by V_{te}, guarantees mass transport.

detected in the apical membrane of epithelial cells of the anterior midgut of *St. aegypti* larvae. At 5 mM, ouabain did not inhibit the capability of intact larvae to alkalize their anterior midgut, nor did it cause isolated and perfused midgut preparations to significantly change their transepithelial voltage or the capacity for luminal alkalization. Onken *et al.* (2009) concluded that Na^+/K^+-ATPase is not of direct importance for strong luminal alkalization in the anterior midgut of larvae *St. aegypti*. Na^+/K^+-ATPase was detected by biochemical tests in homogenized, whole midgut preparations from adult female *An. stephensi*, and, because more than 80% of the total Na^+/K^+-ATPase was sensitive to 4.5×10^{-7} M ouabain, MacVicker *et al.* (1993) concluded that the adult midgut of *An. stephensi* is actively involved in post-blood feeding ion and water regulation.

(g) Conclusions

The high pH in certain regions of the midgut lumen is probably maintained by a carbonate buffer. Hydration of CO_2 produces carbonic acid which dissociates to bicarbonate and, under suitable conditions, to carbonate ions, the earlier steps being catalysed by carbonic anhydrase (Figure 46.9A). Examination of findings from the wide range of experimental techniques used by different investigators led to the development of a provisional model of the process of alkalization in the lumen of the anterior stomach of *St. aegypti* larvae (Onken *et al.*, 2004a). The presence of Na^+ in the haemolymph substitute was essential for maintenance of the lumen negative transepithelial voltage (V_{te}), so the model was drawn to show separate Na^+-independent and Na^+-dependent components (Figure 46.9B).

The principal anions in the alkalization process are HCO_3^- and CO_3^{2-}, the HCO_3^- being derived from cellular metabolism and exchanged for ingested Cl^-. Within the anterior stomach lumen, where the pH can be >10.1, the bicarbonate: carbonate ratio would be close to one. V-ATPase present in the basal membrane of epithelial cells of the anterior stomach mediates transbasal H^+ absorption (into the haemolymph) and generates a driving force for apical transporters, notably an electrophoretic Cl^-/HCO_3^- exchanger, resulting in transapical HCO_3^- secretion. Carbonic anhydrase accelerates the generation of HCO_3^- and H^+, so providing substrates for those transporters.

The luminally negative V_{te} could supply the driving force for the transcellular movement of Na^+ from haemolymph to stomach lumen, and/or the movement of Cl^- in the opposite direction. Altogether, the serotonin-stimulated cell population could mediate transapical HCO_3^- secretion, transbasal H^+ absorption, transepithelial Na^+ secretion and transepithelial Cl^- absorption. Exchange of K^+ from epithelial cells for H^+ from the gut lumen would contribute to the high lumen pH. These processes could all contribute to luminal alkalization under *in vivo* conditions.

The apparent absence of carbonic anhydrase, at any significant titre, from the anterior stomach of *St. aegypti* larvae is not consistent with the model. It has been surmised that it is present in an undetected isoform.

46.5.6 Processing of the δ-endotoxins

(a) Natural and experimental processes

When spores and parasporal bodies of *B. thuringiensis* are ingested by mosquito larvae, they pass through the alimentary canal with the food mass, first becoming exposed to the strongly alkaline condition of the anterior stomach which causes their solubilization. The crystalline parasporal bodies contain a number of δ-endotoxins, the types varying with subspecies, and within the midgut these are processed in two phases. (i) Dissolution of the crystal proteins. This releases mostly **protoxins** but the release of innocuous proteins has also been reported. (ii) Partial proteolysis of the solubilized proteins. The protoxins are subjected to partial cleavage by proteolytic enzymes – either digestive proteinases secreted by the mosquito larva or, less commonly, endogenous proteinases of the bacterial cell. The products of proteolysis are **toxins**, which are proteins of lower molecular mass but which retain a tertiary structure.

A number of preparative procedures are necessary before any analysis of δ-endotoxins can start. In a classic analysis of parasporal crystals from *B. t. israelensis*, cultures were first centrifuged to separate a spore-crystal mixture, which in turn was subjected to differential ultracentrifugation. Incubation of crystals at pH 10.5 and 37°C for 60 min yielded soluble and insoluble fractions. Proteins from both fractions were solubilized by 5 min incubation at 100°C in a reducing solution. Finally, proteins were separated by SDS/PAGE electrophoresis (Thomas and Ellar, 1983a).

Commonly, protoxins are described as 'inactive' while their proteolytic products are described as 'activated toxins'. In fact, due to experimental difficulties, the toxicities of few if any naturally solubilized δ-endotoxins have been assessed under physiological conditions, and it is not known how many protoxins need proteolytic cleavage to become active. For that reason, in this chapter, protoxins are not described as inactive and toxins are not described as activated.

(b) Examples of analytical findings

Cry4A of *B. t. israelensis* could be synthesized by a transfected acrystalliferous strain (HD522), and the 130 kDa protoxin could be obtained by incubating purified parasporal bodies in a solution of 50 mM Na_2CO_3 (pH 10.5) and 10 mM dithiothreitol, and separating the products by SDS/PAGE electrophoresis (Nishimoto *et al.*, 1994). Exposure to gut extract from *Cx. pipiens* larvae converted the protoxin first to a 60 kDa fragment and subsequently to 20 and 45 kDa fragments. The 20 and 45 kDa polypeptides were also produced under *in vivo* conditions. Those two fragments could associate to form a complex of 60 kDa which was toxic to *Cx. pipiens* larvae (Yamagiwa *et al.*, 1999).

After solubilization of **Cry11A** crystals from *B. t. israelensis* by exposure to pH 10.5 and reducing conditions, the 72 kDa protoxin could be processed *in vitro* into 36 and 32 kDa fragments by commercial trypsin, and into 34 and 32 kDa fragments by gut proteases from *Cx. pipiens*. Neither the 36 nor the 32 kDa fragment alone was toxic to *Cx. pipiens* larvae, but a heterodimer of the two fragments produced by co-precipitation was toxic (Dai and Gill, 1993; Yamagiwa *et al.*, 2002, 2004).

The parasporal bodies of *B. t. medellin* contained two main proteins, of 94 kDa (Cry11Bb) and 28 kDa (CytAb), which could be solubilized by exposure to pH 10.6. Treating the protoxin of **Cry11Bb** with trypsin quickly generated a 68 kDa fragment, which in time was transformed to fragments of 30 and 35 kDa. When parasporal bodies were ingested by *Cx. quinquefasciatus* larvae, the protoxin was processed to fragments of 30 and 35 kDa, and after 20 h to one fragment of 20 kDa. Segura *et al.* (2000) postulated that the 30 and 35 kDa fragments remain together in solution and compose the toxic core of the Cry11Bb protein.

Treating solubilized **Cyt1Ab** (28 kDa) from *B. t. medellin* with commercial proteases or larval gut extract produced a 25 kDa fragment, and a similar result was obtained *in vivo* when crystals were ingested by *Cx. quinquefasciatus* larvae. Crystals containing Cyt1Ab were mosquitocidal; the experimentally solubilized and processed toxin was not mosquitocidal but had haemolytic activity (Escobar *et al.*, 2000).

The protoxin of **Cyt2Aa** from *B. t. kyushuensis*, a dimer of 58 kDa, was inactive until processed to the 23 kDa form, either in the larval gut or by proteinase K; further digestion yielded a 20 kDa fragment. Both the 23 and 20 kDa polypeptides were cytologically active. The processing cleaved the intertwined N-terminal arm (including β-strand 1) responsible for dimerization, thereby releasing the active toxin as a monomer. It also removed the C-terminal tail and the α-helix F, uncovering the three-layered core (Section 46.5.8.a).

Certain protoxins can be converted to toxins by endogenous proteinase from within the bacterial cell. This was reported (without supporting evidence) to occur during the solubilization of Cyt1A from *B. t. israelensis* (Al-yahyaee and Ellar, 1995). The experimental conversion of the **Cyt2Ba** protoxin to toxin by an endogenous proteinase deserves mention. The solid remnants of sporulation (crystals, cell debris, spores) in a *B. t. israelensis* culture were separated from the culture

medium by centrifugation and resuspended in buffer. The preparation was divided into two portions, A and B. Portion A alone was subjected to a biphasic separation which yielded a purified crystals fraction. For the purpose of protein solubilization, the pH of both portions (A, now crystals only; B, crystals, debris, spores) was adjusted to 10.5 for a period and then reduced to 8.0. Both portions were then incubated: portion A with addition to the crystal suspension of an exogenous protease (trypsin, chymotrypsin or proteinase K); portion B without such an addition. Subsequent analyses revealed that protease in portion B, necessarily endogenous protease, had degraded the native Cyt2Ba (24 kDa) to a toxin of 22 kDa that possessed haemolytic activity. A similar product was produced in portion A as a result of its incubation with the exogenous proteinase (Nisnevitch *et al.*, 2006).

46.5.7 Cry proteins – topology, binding and pore formation

(a) Topology of Cry proteins

The Cry proteins are toxins present in the parasporal crystals of *B. thuringiensis*. Most are globular, three-domain proteins: i.e. they have folded polypeptide chains that give the whole molecule a rounded shape; and most are formed of three domains (a helix bundle, a β-prism and a β-sandwich), which variably function in receptor recognition, insertion into cell membranes and pore formation.

The Cry toxins from different subspecies of *B. thuringiensis* vary in host specificity. Structural interpretation of their crystalline form, supported by biochemical evidence, revealed that Cry toxins undergo major, irreversible, conformational changes that transform the initially soluble protein bound at the cell surface into a form able to insert into the cell membrane, given the appropriate initial contact with a membrane receptor.

Cry4Ba has the structural details that characterize Cry toxins from the *B. thuringiensis* subspecies that affect Diptera (Figure 46.10).

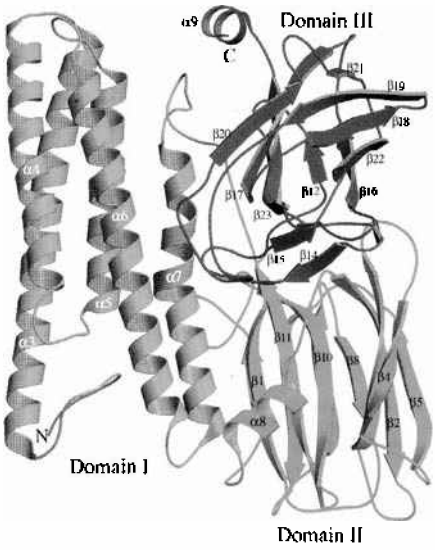


Figure 46.10 Ribbon diagram of the δ-endotoxin Cry4Ba from *Bacillus thuringiensis israelensis*, determined by X-ray crystallography of purified Cry4Ba crystals. (From Boonserm *et al.*, 2005.) C, carboxy terminus; N, amino terminus. Domain I is a helix bundle in which helices α3, α4, α6 and α7 form an arc around α5 (not apparent from this aspect). Domain II is a β-prism of three antiparallel β-sheets. Domain III is a β-sandwich of two antiparallel β-sheets. (Antiparallel: of parallel linear structures, such as polypeptide chains, having directional asymmetry in opposite directions. Prism: a crystal form of which three or more faces are parallel to one axis.) (See Glossary, Appendix 3, for α-helix and β-sheet.)

Initially, the N-terminal **domain I** is a bundle of seven α-helices in which a relatively hydrophobic central helix (α5) is encircled by six amphipathic helices (i.e. having one end hydrophilic and the other hydrophobic). After proteolysis during crystallization, helices α1 and α2 are absent, and domain I is reduced to a bundle of five helices, in which helices α3, α4, α6 and α7 are arranged in an arc around α5. The Arg-235 and Arg-203 residues, which are located in the interhelical 5/6 loop of domain 1, are solvent exposed and so may be susceptible to tryptic cleavage. As in other Cry toxins, **domain II** is a β-prism of three antiparallel β-sheets. The apical loops of domain II are the most variable part of the many Cry toxins, and those of Cry4Ba are the shortest known for Cry toxins.

Domain III, which is thought to protect the structural integrity of active toxin molecules against further proteolytic cleavage, and to promote membrane permeabilization, is a β-sandwich of two antiparallel β-sheets. A conformational change, possibly triggered by receptor binding, is thought to involve an umbrella-like opening between the helix-4,5-hairpin and the remaining helices, and between the helical domain and the two sheet domains (Li *et al.*, 2001; Angsuthanasombat *et al.*, 2004; Boonserm *et al.*, 2005).

Cry4Aa comprises three domains, which are of a generally similar structure to those of Cry4Ba, but the two proteins differ in certain details; for example, domain 1 of Cry4Aa toxin is a bundle of seven amphipathic helices (Boonserm *et al.*, 2006a).

(b) Binding characteristics of Cry proteins

Immunodetection studies on the midgut of *An. gambiae* larvae revealed that the three Cry toxins of *B. t. israelensis* (Cry4A, Cry4B, Cry11A) bind to the plasma membrane of midgut epithelial cells, but to different extents in the different midgut regions. Binding was strongest to microvilli in the posterior stomach region; it was weaker to microvilli in the gastric caeca, and at very low intensity to microvilli in the anterior stomach region (Ravoahangimalala and Charles, 1995). In *Cx. pipiens* larvae, Cry4A bound to the long microvilli of epithelial cells of the gastric caeca and posterior stomach, but not to the shorter microvilli of the anterior midgut cells. This binding was almost abolished in the presence of excess unlabelled Cry4A, suggesting that it was specific. It was not affected by pH (Yamagiwa *et al.*, 2001). In larvae of *St. aegypti*, *Cx. quinquefasciatus* and *An. albimanus* that had ingested the solubilized protoxin Cry11Bb (of *B. t. medellin*) and that showed toxic symptoms, activity was detected mostly on microvilli of the posterior stomach and gastric caeca cells, but also in the Malpighian tubules (Ruiz *et al.*, 2004).

The binding of a δ-endotoxin to microvilli was investigated with Cry1C from *B. thuringiensis aizawai*, a subspecies that is toxic to lepidopteran larvae and culicid larvae. First, the *cry1C* gene from *B. t. aizawai* was transfected into acrystalliferous *B. t. israelensis*, from which Cry1C was purified; it proved toxic to *St. aegypti* larvae and to three out of four lepidopteran larvae tested (Smith *et al.*, 1996; Abdul-Rauf and Ellar, 1999b). Then 'brush border membrane vesicles' were prepared from the microvilli of *St. aegypti* larvae by a sequence of partial homogenization of whole larvae, followed by an Mg^{2+} precipitation-differential centrifugation cycle, to yield fuzzy-coated membrane vesicles of different sizes (Abdul-Rauf and Ellar, 1999a,b). Binding experiments with ^{35}S-labelled Cry1C and vesicles revealed a single class of high-affinity binding sites with a dissociation constant (K_d) of 25–27 nM and a maximum binding capacity of 25–27 pmol mg^{-1} protein. About 30% of the toxin that was bound to vesicles could be displaced by unlabelled toxin. The toxicity of Cry1C to *St. aegypti* larvae correlated with both binding affinity and the ability to bind irreversibly (Abdul-Rauf and Ellar, 1999a,b).

In the anti-lepidopteran toxins Cry1Ab and Cry1Ac, domains II and III both play a major role in binding to putative receptors and determining toxin specificity. Exchanging domains between closely related toxins resulted in toxin hybrids with altered specificities, and showed that toxicity to the insect host followed the movement of domain III (de Maagd *et al.*, 1996a,b).

Experimental results suggested that domain III of Cry4Ba is involved in receptor recognition and that this determines host specificity. Mutations that affected loop 3 in Cry4Ba induced significant toxicity towards *Cx. pipiens* and *Cx. quinquefasciatus*, against which that toxin shows no natural activity. Mutations in loops 1 and 2 abolished the natural toxicity of Cry4Ba towards *St. aegypti* and *An. quadrimaculatus* (Abdullah *et al.*, 2003). With Cry11Aa, the requirement for loop α8 in domain II for interaction with a midgut receptor from *St. aegypti* was demonstrated by site-directed mutagenesis (Fernández *et al.*, 2005). Cry toxins from the *B. thuringiensis* subspecies that are variably toxic to Diptera, Coleoptera or Lepidoptera differ most in domain II.

(c) Receptors of Cry proteins

Cry toxins bind to specific proteins (receptors) in the plasma membrane of epithelial cells of the host midgut. Under *in vivo* conditions, labelled Cry4A bound specifically to apical microvilli on epithelial cells of the gastric caeca and posterior midgut of *Cx. pipiens* larvae. Ligand-blotting analysis of proteins that had been isolated from brush border membrane vesicles prepared from *Cx. pipiens* larvae showed specific binding between proteins of 18–30 kDa and labelled Cry4A (Yamagiwa *et al.*, 2001).

Cry toxin binding has been investigated in most detail for the anti-lepidopteran Cry1A toxins, for which the known receptors are membrane glycoproteins – namely aminopeptidases, alkaline phosphatase, and a protein of the cadherin family (a family of calcium-dependent, cell adhesion proteins that interact in a homophilic manner, i.e. an adhesion protein in one cell binding to an identical protein in another cell) (Knight *et al.*, 1994; Vadlamudi *et al.*, 1995; Jurat-Fuentes and Adang, 2004).

A cadherin detected in the midgut of *Anopheles gambiae* larvae by PCR was named AgCad1, and was localized by immunochemistry predominantly to microvilli in the posterior midgut. The localization of Cry4Ba binding was determined by the same technique, which showed toxin-bound microvilli in the posterior midgut. Preparations of larval brush border membrane fractions contained AgCad1, while Cry4Ba toxin bound the same-sized protein on blots of those fractions. On the basis of these findings, Hua *et al.* (2008) concluded that AgCad1 is probably a Cry4Ba receptor.

In a number of cases, proteins that had been extracted from brush border membranes of culicid larvae, and that were capable of specific binding to Cry4B and Cry11A from *B. t. israelensis*, had molecular masses of or close to 65 kDa. Krieger *et al.* (1999) isolated two such proteins, of 65 and 62 kDa, from *St. aegypti* larvae, both of which bound reversibly to Cry4B and Cry11A. The two toxins competed for binding to the two putative receptors. Buzdin *et al.* (2002) reported that a 65 kDa protein was the only component of brush border membranes from the midgut of *St. aegypti* larvae capable of specific binding to Cry4B and Cry11A. It did not bind to lepidopteran Cry toxins, possibly because it lacked the leucine aminopeptidase activity that is characteristic of toxin-binding proteins from the equivalent membranes of caterpillars.

Proteins of 65 and 57 kDa that had been isolated from apical membrane of midgut epithelial cells from *An. stephensi* larvae bound specifically to Cry4B and Cry11A. They shared most of the characteristics of the putative midgut receptors in *St. aegypti* larvae. The extract of brush border membrane proteins showed high alkaline phosphatase activity (Dronina *et al.*, 2006).

A 148 kDa protein in brush border membrane vesicles prepared from larval midguts of *An. stephensi* larvae bound strongly to a solubilized protein of 65 kDa obtained from Cry11Aa crystals from *B. t. israelensis*. Membrane preparations from larval midguts of *Tipula oleracea* (Diptera, Tipulidae) and *Manduca sexta* (Lepidoptera, Sphingidae) bound to the same 65 kDa protein. Ingestion of Cry11Aa crystals was fatal to the larvae of both dipteran species but not to the lepidopteran larvae (Feldmann *et al.*, 1995). A 100 kDa protein with aminopeptidase activity isolated from brush border membrane from *An. quadrimaculatus* larvae, in its native state, bound to Cry11Ba and was considered a putative receptor. (Cry11BA is known from *B. t. jegathesan* but not from *B. t. israelensis*; Table 46.4.) The 100 kDa protein did not bind to Cry2Aa, Cry4Ba or Cry11Aa, three toxins that are pathogenic for *An. quadrimaculatus* larvae (Abdullah *et al.*, 2006).

(d) Pore formation by Cry toxins

There is strong circumstantial evidence for the existence of pores formed of Cry proteins, but little physical evidence of their nature. The rate-limiting steps in pore formation are receptor binding, which is reversible, and membrane insertion, which is irreversible.

Knowledge of the crystal structure of Cry4Ba from *B. t. israelensis*, coupled with mutagenesis and biophysical data for this protein, enabled development of a theoretical explanation of the means of pore formation. Domain I was thought to

be involved in membrane insertion and pore formation. As in other Cry toxins, the helical domain I is connected to the β-sheets of domain II by a long, hinged link, and the unfolding of the protein around this link is necessary for pore formation (Figure 46.10). Modelling of Cry4Ba suggested that a conformational change exposes a relatively non-polar helix hairpin from domain I to initiate membrane penetration. Of the available helices, the α4-loop-α5 region is the most hydrophobic and is thought to insert into the membrane. The remaining helices rearrange to open on the membrane surface like the ribs of an umbrella (Boonserm *et al.*, 2005). The long loop connecting α4 and α5 contains a unique disulphide bond and a proline-rich region (Angsuthanasombat *et al.*, 2004). Evidence for the involvement of this helix loop in binding and pore formation came from studies of mutant toxins; substitution of Asn-166 and Tyr-170 with alanine in Cry4B (or of Tyr-202 with alanine in Cry4A), with a consequent loss of aromaticity, caused a complete loss of mosquito larvicidal activity (Kanintronkul *et al.*, 2003; Pornwiroon *et al.*, 2004). In Cry4Aa, the α4–α5 loop contains proline residues at positions 193, 194 and 196; they are flanked at positions 192 and 199 by cysteine residues that form a disulphide bridge that restricts the flexibility of this segment (Boonserm *et al.*, 2006a).

Cry4Ba inserts spontaneously into receptor-free lipid bilayers. Use of atomic force microscopy showed that it inserted as a self-assembled tetrameric structure rather than as a single monomeric molecule. Insertion led to the formation of pore-like structures with fourfold symmetry, suggesting that tetramers were the preferred oligomerization state (Puntheeranurak *et al.*, 2005).The insertion of Cry4Ba into lipid films of mixed composition was enhanced by the presence of cholesterol as a constituent (Kanintronkul *et al.*, 2005).

46.5.8 Cyt toxins – topology and binding characteristics

The Cyt δ-endotoxins are a family of some nine known members, specifically toxic *in vivo* to dipteran larvae. Each toxin consists of a single, folded domain with a molecular mass of 22–25 kDa.

(a) Topology of Cyt proteins

The crystal structure of Cyt2Aa1 from *B. t. kyushuensis* was determined by isomorphous replacement using heavy-atom derivatives. In its protoxin form, Cyt2Aa1 is a dimer linked by the intertwined N-terminal β-strands into a continuous 12-stranded β-sheet (Figure 46.11A). Proteolytic processing cleaves the intertwined β-strands to release the Cyt2Aa1 toxin as a monomer, and removes the C-terminal tail to uncover the three-layered core. The toxin is a single-domain polypeptide of α/β architecture, but with a novel connectivity comprising two outer layers of α-helix hairpins wrapped around a mixed β-sheet (Figure 46.11B). Structural mutations revealed that segments forming the β-sheet are responsible for both membrane binding and pore formation (Li *et al.*, 1995, 1996b, 2001).

Cyt1Aa from *B. t. israelensis* shares 39% sequence identity with Cyt2Aa from *B. t. kyushuensis*, and the two polypeptides are expected to be very similar in structure (Koni and Ellar, 1993).

Alignment of the amino acid sequences of six Cyt proteins (Cyt1Aa1, Cyt1Aa3, Cyt1Ab1, Cyt1Ba1, Cyt2Aa1, Cyt2Ba1) revealed four blocks of amino acids with highly significant similarity scores. These were: (i) helix A; (ii) the loop after helix D plus strand 4; (iii) strands 5 and 6; and (iv) strand 6a and the following loop (Butko, 2003).

(b) Binding characteristics of Cyt proteins and membrane lipids

Cyt1A from *B. t. israelensis* bound strongly to apical microvilli on epithelial cells in the posterior stomach of *An. gambiae* larvae, but less strongly to microvilli on cells of the gastric caeca, and less strongly still to microvilli in the anterior stomach region. When purified crystals of Cyt1A and Cry4A were fed together to *An. gambiae* larvae, immunodetection showed that both had bound to microvilli on cells of the posterior stomach and gastric caeca, but not to microvilli on cells of the

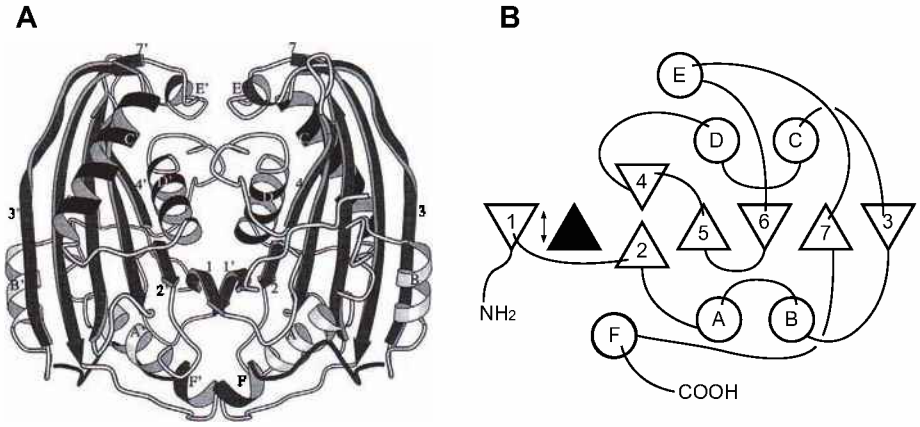


Figure 46.11 Diagrams showing the molecular structure of the δ-endotoxin Cyt2Aa1 (formerly CytB) from *Bacillus thuringiensis kyushuensis*. (From Li *et al.*, 1996b.) **A.** Ribbon drawing of the dimer viewed along the central pair of strands with the dimer axis vertical. **B.** Topology diagram of a Cyt2Aa1 monomer. α-Helices are represented by circles, and β-strands by triangles which point up or down depending on whether the N terminus of the strand points out of the page or into it. The α-helices are in hairpin conformation, and form two outer layers, A–B and C–D, that flank a sheet of β-strands. The unconnected, shaded triangle (1′) represents the interleaved β1 strand (β′) from the other monomer generated by a twofold rotation about the axis indicated. The α-helix and β-strand (see Glossary, Appendix 3) are the basic elements of the hydrophobic core of a globular protein.

anterior stomach (Ravoahangimalala *et al.*, 1993; Ravoahangimalala and Charles, 1995).

Experimental evidence indicated that Cyt toxins do not enter cell membranes by first binding to protein receptors but by interacting with membrane lipids. Early studies showed that toxins from *B. t. israelensis* caused rapid cytolysis of cells from insect cell lines and haemolysis of mammalian erythrocytes. The determinants for these activities were: (i) the nature of the 'lipid polar head group' (i.e. that part of a polar lipid molecule that expresses its polar character); and (ii) the presence of unsaturated fatty acyl chains. Binding ability was found in the phospholipids phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, provided they contained unsaturated fatty acids. Preincubation with a tenfold excess of phospholipids from cultured *St. albopicta* cells neutralized the toxin (Thomas and Ellar, 1983a,b).

Cyt1Aa bound to phospholipids that contained unsaturated fatty acids. The cytolytic activity of Cyt toxins was inhibited by interaction with added unsaturated phospholipids (Thomas and Ellar, 1983b; Weinstein *et al.*, 1988; Knowles *et al.*, 1989, 1992). Studies of Cyt2Aa from *B. thuringiensis darmstadiensis* showed that both the solubilized crystal protein and the toxin were cytolytic to a range of eukaryotic cells. Cytotoxicity of the toxin to *St. albopicta* cells was inhibited by phospholipids containing unsaturated fatty acids with a single double bond in either *cis* or *trans* orientation, but not by phospholipids containing saturated fatty acids (Drobniewski and Ellar, 1989). Treatment of erythrocytes with high levels of phospholipase D increased their susceptibility to Cyt1Aa from *B. t. israelensis*, revealing the need for an unsaturated chain at the *syn*-2 position of membrane phospholipids for haemolytic activity (Gill *et al.*, 1987b).

Cyt toxins are known only from subspecies of *B. thuringiensis* that are active against dipterans, and they interact preferentially with unsaturated phospholipids. Citing Fast (1966) and Jenkin (1976), Li *et al.* (1996b) commented that dipterans have a higher proportion of their phospholipids unsaturated than do lepidopterans or coleopterans, which are not susceptible to Cyt toxins. In most Diptera, phosphatidylethanolamine accounts for about 50% of the phospholipids, almost twice as

much as does phosphatidylcholine. The predominance of phosphatidylethanolamine is associated with a higher proportion of unsaturated fatty acids in the phospholipids than is found in other insects.

Like the first step in the action of a number of poisons, several toxin molecules assemble on the cell membrane. Evidence that this may be the case with Cyt1Aa was obtained in experiments with isolated Malpighian tubules of *Rhodnius* (a triatomid bug), the fluid secretion of which was inhibited by Cyt1Aa. An inverse, sigmoid relationship was found between toxin concentration and time to failure of secretion. This suggested that toxin molecules approach the cell as monomers, and then form an oligomeric complex on the cell membrane. Further experiments discounted the possibility that toxin molecules had aggregated before their addition to Malpighian tubule preparations (Maddrell, 1988, 1989).

Other investigators obtained similar results. When cultured *St. albopicta* cells were exposed to Cyt1Aa, the toxin bound irreversibly to their cell membranes in a concentration-dependent manner. A 2- to 3-fold increase in the rate of binding occurred after the amount of membrane-bound toxin had reached *c.* 3.5 fmol 2×10^{-5} cells, at which stage the toxin molecules began to form aggregates of *c.* 400 kDa at the cell membrane. The formation of these aggregates appeared to be essential for subsequent cell lysis. Monoclonal antibodies raised against the native form of the toxin blocked most binding, and produced evidence suggesting that the C-terminal domain of Cyt1Aa is involved in binding to cell membranes (Chow *et al.*, 1989).

(c) Pore formation by Cyt proteins

Cyt2Aa from *B. t. kyushuensis* formed cation-selective channels in planar lipid bilayers and released glucose from liposomes (Knowles *et al*, 1992).

Knowledge of the crystal structure of Cyt2Aa, a haemolytic toxin from *B. t. kyushuensis*, led to hypotheses on its mechanism of attachment and pore formation. In brief, it was proposed that toxin molecules approach the cell membrane and bind as monomers, the β-sheet being strongly implicated in both binding to phospholipids and pore formation. The strands in the β-sheet are long enough to span the bilayer. A conformational change was postulated to follow contact with a cell membrane, when the α-helix pair C–D is lifted in a hinge movement off the β-sheet to lie on the membrane surface (Figure 46.11B). This is followed by insertion of the underlying long amphiphilic β-strands (β5, β6 and β7) into the bilayer, with the loop between β5 and β6 orientated towards the cell interior. The α-helices are exposed on the membrane surface, while the rearranged β-strands contribute to the formation of an oligomeric transmembrane pore.

Cyt2Aa exists in three different configurations, water-soluble monomer, membrane-inserted monomer and membrane-inserted oligomer. Oligomerization occurs when toxin molecules in close proximity bind to membrane receptors. Transmembrane pores form when the monomers reach a critical number, and the inserted β-strands from six monomers associate to form an oligomeric pore (Li *et al.*, 1996b, 2001; Promdonkoy and Ellar, 2000, 2005). In one working model, the putative Cyt2Aa pore comprises six toxin molecules assembled like an open umbrella. The β-strands 5–7 of each molecule span the membrane and form the handle of the umbrella, while the top of the umbrella is formed by the α-helices splayed on the membrane surface (unpublished data of Promdonkoy and Ellar, cited by Butko, 2003).

Cyt1Aa from *B. t. israelensis* was reported to form cation-selective channels in planar bilayers (Knowles *et al.*, 1989), a finding that has been questioned (Section 46.5.9.a). Single molecules of Cyt1Aa from *B. t. israelensis* are too short to span a bilayer, and it is generally agreed that molecules of Cyt1Aa must aggregate on the membrane in a different manner from those of Cyt2Aa to exert their cytolytic action.

46.5.9 Modes of action of δ-endotoxins

Two modes of action have been postulated for the destructive effects of δ-endotoxins on the midgut

epithelium of susceptible insect larvae – colloid-osmotic lysis and a detergent-like action.

(a) *Colloid-osmotic lysis*

It is thought that many δ-endotoxins form membrane pores in cells of the larval midgut epithelium, so creating leakage channels and leading to cell lysis and eventually death of the larva. Both Cry and Cyt toxins have been shown to form pores in planar lipid bilayers (Sections 46.5.7.d, 46.5.8.c). Most studies of the characteristics of δ-endotoxin-induced pores have made use of cultured insect cells.

Knowles and Ellar (1987) postulated that the cytolytic consequences of pore formation result from 'colloid-osmotic lysis', i.e. an equilibration of ions through the pores that results in a net inflow of ions, an osmotically driven influx of water, cell swelling and eventual lysis. Cell lysis breaches the epithelial barrier between gut lumen and haemocoel. Observations of cell swelling preceding lysis were consistent with this theory.

Wolfersberger (1992) described the effects of δ-endotoxins on lepidopteran larvae in biophysical terms. When toxin molecules insert into the apical plasma membrane, cation-conducting pores are formed and the cation permeability of the membrane increases greatly. The large electrical potential difference ($\Delta\Psi$) normally maintained across this membrane collapses, and this allows for redistribution of cations between the midgut lumen and cell cytoplasm. The most devastating consequence of the collapse is loss of the force that energizes maintenance of the 10^3-fold H^+ activity gradient across the apical membrane of midgut cells. The resulting increase in cytoplasmic pH, from ~pH 7 to ~pH 10, is large enough to produce the symptoms seen in histopathological studies.

Early studies with lytic δ-endotoxins appeared to demonstrate the involvement of pores. Thus, *St. aegypti* cells preloaded with radio-labelled molecules of different sizes, and treated with δ-endotoxin, showed a graded release with smaller markers emerging before the larger. Addition to the incubation medium of osmotic protectants such as raffinose, with a viscometric radius greater than the radii of the pores, increased the external osmotic pressure, opposed the influx of water and slowed the onset of lysis (Knowles and Ellar, 1987; Drobniewski and Ellar, 1988, 1989; Ellar, 1997).

When planar bilayers were exposed to Cyt1Aa, the toxin formed cation-selective channels that were permeable to K^+ and Na^+, showing very fast cooperative opening and closing. The channels were impermeable to N-methylglucamine and Cl^-, and channel opening was greatly reduced in the presence of divalent cations (Ca^{2+} and Mg^{2+}) (Knowles *et al.*, 1989). Butko (2003) commented that the results from such experiments might vary greatly with toxin concentration or with duration of exposure, leaving the argument open.

Estimates of pore size were obtained by measuring the half-time of lysis of toxin-treated cells suspended in a cell culture medium and exposed to different solutes of known viscometric radius. Approximate pore radii were <0.6 nm for pores induced by Cry toxins from *B. t. kurstaki*, and 0.6–1.0 nm for pores induced by Cry toxins from *B. t. aizawai* and *B. t. israelensis*. Pores of those dimensions would allow equilibration of ions but not leakage of cytoplasmic macromolecules (Knowles and Ellar, 1987).

(b) *Detergent-like activity*

The affinity of certain Cyt toxins for phospholipids with fatty acyl side chains, and the cytolytic and haemolytic actions of those toxins (Section 46.5.8.b), led to the idea that they cause a detergent-like rearrangement of membrane lipids, with aggregates of toxin molecules on the membrane surface disordering the membrane lipids and producing temporary packing faults.

An investigation into whether Cyt1A from *B. t. israelensis* forms membrane pores or has a detergent-like action tested two postulates. (i) If, in the presence of lipid, Cyt1A self-assembles into SDS-insoluble protein-lined pores, then SDS-PAGE electrophoresis of Cyt1A in the presence of lipid should yield a protein band with a molecular mass of the oligomeric pore, possibly accompanied by a

few bands of lower molecular mass corresponding to intermediate aggregates. (ii) If Cyt1A has a detergent-like action, then SDS-PAGE electrophoresis in the presence of lipid should yield protein aggregations of higher molecular mass. In the absence of lipid, Cyt1A migrated as a monomer of 24 kDa. In the presence of lipid, Cyt1A migrated to produce a laddered pattern, showing a distribution of molecular masses from small oligomers to large polymers, some of the latter being too large to enter the gel. A technique that measured the diffusion rate of fluorescently labelled particles showed that, when Cyt1A was added to a suspension of lipid vesicles, the vesicles transformed into smaller bodies, consistent with a detergent-like action. Manceva *et al.* (2005) concluded that these findings gave strong support to the detergent model of Cyt1A action. However, the detergent model could not accommodate such features of Cyt1A action as the toxin's specificity for certain insects and its high affinity for unsaturated lipids.

Earlier experiments with liposomes also provided evidence of a detergent-like action. Addition of toxins from *B. t. israelensis* to phospholipid liposomes caused a decrease in turbidity, suggesting that smaller lipid structures had formed (Thomas and Ellar, 1983b). Exposure of phospholipid liposomes to δ-endotoxins from three subspecies of *B. thuringiensis* caused a rapid increase in light scattering, while similar treatment of multilamellar liposomes loaded with radioactive markers led to release of 70% of the markers. It was postulated that five conserved, hydrophobic domains in the N-terminal half of the high molecular mass toxins (of 135–145 kDa) can interact with lipid bilayers, and that they play a major role in the insertion of those toxins into target membranes (Haider and Ellar, 1989).

The effects of one Cry toxin on larval midgut *in vivo* were consistent with a detergent-like action. Exposure of *St. aegypti* larvae to Cry4Ba for 24 h seriously damaged the midgut in some larvae, but some other larvae survived and appeared normal. Examination by scanning electron microscopy of midguts from apparently unharmed larvae showed that the cells of the anterior and posterior stomach regions were not visibly affected, but that holes of 1–7 μm diameter and blisters 17 μm wide appeared in the apical plasma membrane of cells of the transitional region (Clark *et al.*, 2005).

(c) Conclusions

Further work is needed to establish whether the damaging effects of individual δ-endotoxins result from pore formation or from a detergent-like action. Possibly, certain of the toxins have one mode of action and other toxins the other. Further, it may be that temporary packing faults produced by a detergent-like action are simply a grosser form of pore and also cause colloid-osmotic lysis.

46.5.10 Pathogenesis

(a) Pathogenesis in mosquitoes

Infection of mosquito larvae by *B. thuringiensis* starts with the ingestion of parasporal bodies and endospores that have dispersed from cadavers into the mosquito's aquatic habitat. Particles suspended in the water column are carried to the pre-oral cavity in currents generated by the beating mouthparts, as are submerged deposits that actions of the mouthparts render particulate and resuspend (Volume 1, Section 4.3). Mosquito larvae remain near and feed more actively on food sources from which phagostimulants diffuse into the water, and they will ingest spores and parasporal bodies of *B. t. israelensis* that are mixed with acceptable food matter.

Suspended latex particles have often been used in studies of larval feeding mechanisms. The time course of the action of toxins on mosquito larvae was revealed in measurements of the rates at which feeding larvae removed 2 μm latex particles from suspension in the presence or absence of *B. t. israelensis*. With yeast extract added as a phagostimulant, *St. aegypti* larvae fed uninterruptedly for 40 min, during which time 38% of latex particles were removed, whether the pathogen was present or not. In the absence of the pathogen, particle clearance

continued at the same rate for 180 min or more, but if the pathogen was present, feeding stopped within 40–80 min of exposure (Figure 46.12). After 90 min exposure very few larvae were still feeding; others showed symptoms of intoxication, such as weak, ineffective swimming movements.

In the same experiment, *Culex quinquefasciatus* larvae were affected more rapidly. Their feeding rate declined within 20 min of exposure to *B. t. israelensis*, and thereafter there was little clearance of particles by the treated larvae. *Anopheles albimanus* larvae, which are surface feeders, cleared particles from suspension at a much slower rate, but even so most stopped feeding between 40 and 80 min after exposure (Aly, 1988).

Fourth-instar larvae of *Aedimorphus vexans* fed readily on *B. t. israelensis*, whether in the form of suspended particles or incorporated into 1.5 mg food pellets on the substratum at a depth of 5 cm. Except at high pathogen densities, the death rates were much lower among larvae feeding on suspended particles than among larvae feeding on the food pellets (Aly, 1983). Varying the dosage of *B. t. israelensis* suspensions affected feeding and survival rates. When fourth-instar *Am. vexans* larvae were exposed to a spore–crystal suspension of 3×10^3 spores ml^{-1}, all were alive and still feeding after 5 h, but all had stopped feeding by 15 h, at which time 58% were dead. Most larvae exposed to 10^4 spores ml^{-1} stopped feeding within 3 h exposure; at 5 h the death rate was 15% and at 15 h it was 100% (Aly, 1985).

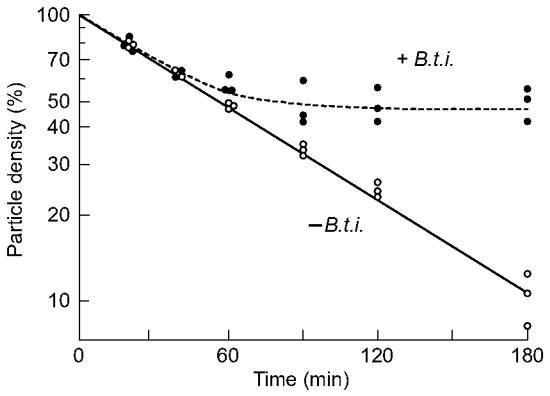


Figure 46.12 Removal of 2 μm latex particles from suspension by *St. aegypti* larvae, feeding in the presence or absence of lethal doses of *Bacillus thuringiensis israelensis* (*B.t.i.*). (After Aly, 1988.) Batches of 120 fourth-instar larvae were placed for 180 min in 100 ml distilled water containing 0.5% yeast extract as phagostimulant, latex microspheres in suspension and lyophilized *B. thuringiensis* in suspension at LC_{95}. Particle density is expressed in relation to the density before addition of larvae.

Toxins that are released by solubilization and proteolysis in the midgut lumen of mosquito larvae bind to the microvilli of the epithelial cells. Fifteen minutes after *An. gambiae* larvae started to feed on a high concentration of purified parasporal crystals from *B. t. israelensis*, immunohistochemical staining revealed binding of Cry11A and Cyt1A toxins to the microvilli of the gastric caeca and posterior stomach (Ravoahangimalala *et al.*, 1993). Thirty minutes or more after the ingestion of crystals from *B. t. israelensis* by *St. aegypti* larvae, large spaces appeared between adjacent midgut epithelial cells, and spaces appeared also within the basal labyrinth of those cells. After 1 h, epithelial cells swelled from their apical surface, the microvilli shrank and disappeared, the endoplasmic reticula disintegrated and the mitochondria lost their structure. Subsequently, a few cells ballooned out into the gut lumen and cell lysis occurred (Charles and de Barjac, 1983). Exposure of second-instar *St. aegypti* larvae to Cry4B led to lysis of midgut cells. Cry11A had similar effects but was less potent (Zalunin *et al.*, 2002).

Spores of *B. t. israelensis* germinate within the midguts of mosquito larvae, whether live, moribund or dead. After infected larvae of *Am. vexans* or *St. aegpti* had died, vegetative cells multiplied in the midgut lumen, bounded by the peritrophic matrix. About 1 day after death, dissolution of the larval tissues led to the distribution of pathogen cells throughout the body. The large *B. thuringiensis* cells appeared relatively few among the masses of other bacteria also developing in the cadavers, which 1 day after death each contained up to 10^7 bacteria. Nevertheless, the pathogen was able to multiply in the cadavers in competition with other bacteria (Aly, 1985).

(b) Pathogenesis of Cereus Group species in mammals

The pathogenicity of *B. thuringiensis* to mammals cannot be considered without reference to that of other Cereus Group species (Section 46.6.1.a), three of which are of medical or veterinary importance. Cereus Group species possess a number of virulence genes, including three non-haemolytic enterotoxin genes (*nheABC*) and two type III haemolysin genes, among others. The type forms of *B. cereus* and *B. thuringiensis* do not possess the toxin genes of *B. anthracis*, detailed below (Han *et al.*, 2006), but there is evidence that conjugation between lineages of *B. anthracis*, *B. cereus* and *B. thuringiensis* has occurred (Section 46.5.1.b).

Bacillus anthracis is an obligate pathogen of mammals, especially ungulates, and is the aetiological agent of anthrax, an acute fatal disease. The endospores can survive in the soil for years, and ruminants may consume them as they graze on low herbage. It is surmised that grazing animals contract anthrax by ingesting spores together with spiky leaves, which cause lesions in the gastrointestinal tract (Turnbull *et al.*, 1998).

Once inside a host, the resistant spores germinate, changing into growing and dividing vegetative cells. The vegetative cells form capsules of poly-D-glutamic acid, which have a negative charge that inhibits macrophages from destroying them. Different interactions between spores and macrophages have been reported, possibly due to the strain characteristics of pathogens and experimental hosts (Moayeri and Leppla, 2004). The genes encoding the major anthrax toxins and the poly-γ-D-glutamic acid capsule are located on two plasmids, pXO1 and pXO2, respectively, both of which are required for full virulence. Among Cereus Group species, only *B. anthracis* has the toxin genes *pag*, *lef* and *cya* encoded on pXO1, and the *cap* gene encoded on pXO2 (Han *et al.*, 2006). Anthrax toxin, which functions in all stages of infections, consists of three polypeptides: a protective antigen (PA), which binds to cellular receptors and which transports lethal factor (LF, a protease) and oedema factor (EF, an adenyl cyclase) into cells.

Anthrax infections in humans most commonly result from contact with diseased animals or infected products, such as animal hides. Inhalation of airborne spores is another means of infection, but the number of spores required for infection by that route is high (Peters and Hartley, 2002). However, systemic infection in humans resulting from inhalation has a mortality rate of almost 100%. In studies at three contaminated sites in Etosha National Park, Namibia, the numbers of *B. anthracis* spores carried downwind over distances of one to 18 metres were far below those required to infect animals by the inhalation route while in transit near or across a carcass site (Turnbull *et al.*, 1998).

Bacillus cereus is present almost everywhere, and is frequently isolated from soil. It is the causative agent of several pathogenic conditions in humans, most commonly food-borne, gastrointestinal infections aggravated by the release of enterotoxins (Lund and Granum, 1997). It also ranks as a leading cause of endophthalmitis, i.e. infection within the eye, following penetrative injury or contamination during surgery (Callegan *et al.*, 1999). Samples taken from human patients sometimes yield isolates of both *B. cereus* and *B. thuringiensis*, e.g. patients with periodontal infections. Strains of *B. cereus* isolated from patients with periodontitis showed a marked clonality (Helgason *et al.*, 2000).

In a number of cases in which *B. cereus* had been identified as the aetiological agent of a severe infection, genomic analysis of the isolates showed them to possess genetic elements almost identical with those of *B. anthracis*. Their genomically intermediate nature was revealed when they were included in phylogenetic analyses of many Cereus Group isolates. The resultant dendrograms showed them interspersed between the lineages of different species (Figure 46.1; Section 46.5.1.a). The identity of these pathogenic *B. cereus*-like isolates must remain uncertain while the species status of *B. cereus*, *B. anthracis* and *B. thuringiensis* remains in flux.

Of 50 strains of ***B. weihenstephanensis*** isolated from pasteurized milk or 'lab-heated raw milk', six were highly cytotoxic by Vero cell assay. Up to three enterotoxins were produced by over 50% of tested strains (Stenfors *et al.*, 2002).

46.5.11 Occurrence in nature

Little is known of the natural history of *Bacillus thuringiensis israelensis*, but something of it can be inferred from knowledge of other Cereus Group species.

(a) Occurrence of Cereus Group species in soil

Endospore formers are among the most widespread of all bacteria, and can be isolated from almost all habitats, possibly as a result of the dispersal of spores by wind. In British Columbia, spores of *B. thuringiensis kurstaki* were present in a high percentage of bulk air samples obtained both within and away from a zone where it was later to be used in crop spraying (de Amorim *et al.*, 2001). In the Negev Desert of Israel, spores of *B. t. israelensis* were said to be transported away from the dried up pools where they originated, carried with particles of loess during dust storms (Section 46.5.11.h).

In some cases, the sampled bacteria are present only as dormant spores in a hostile environment and are not from a self-perpetuating population. Examples include thermophiles recovered from the cold depths of the seabed, and alkaliphiles that can grow only at pH > 9 but that were recovered from normal soils. The data necessary to establish the occurrence of a species in a locality should include the proportions that are present as vegetative cells and as spores (Priest and Grigorova, 1990; Priest and Dewar, 2000).

Reports that all six Cereus Group species have been isolated from soil and that certain of them are distributed very widely need qualification. Frequently, samples are heat treated to destroy vegetative cells, and it cannot be certain that wild-found spores would germinate in the conditions obtaining where they were found. Isolates of *B. thuringiensis* have been reported from most geographical regions, but usually the samples were heated. In one widespread survey, 10 g samples were taken at depths of 2–5 cm below the soil surface from a wide range of habitats in 30 countries. From analyses of the isolates from 1115 soil samples (n = 9776), 70.4% of the samples yielded at least one isolate of *B. thuringiensis*. Of 502 of those isolates tested against *Cx. pipiens* larvae, 22.7% were toxic. Unfortunately, the samples were first subjected to 'acetate selection' to inhibit the germination of *B. thuringiensis* spores, and then heated to a temperature that would eliminate growing cells and non-spore-forming bacteria (Martin and Travers, 1989). The results from investigations in which samples were heat treated are not necessarily incorrect; vegetative cells of the species reported may have been present. Unfortunately, there is often an element of doubt.

The geographical distribution of self-perpetuating populations of a species is limited by the range of ambient temperatures that its vegetative stage can tolerate. In terms of optimum temperatures, bacterial species are classified into three groups: (i) thermophiles, which have an optimum for growth above 45°C; (ii) mesophiles, which have an optimum for growth between 20°C and 45°C; and (iii) psychrotolerants, which can grow at low temperatures, but have an optimum temperature for growth above 20°C. Thus, growth within the temperature range of <7°C to 38°C is characteristic of the psychrotolerant *B. weihenstephanensis*, whereas the mesophilic *B. cereus* and *B. thuringiensis* can grow at temperatures from >7°C to 46°C.

Thermal type governed the distribution of *B. cereus*, *B. thuringiensis* and *B. weihenstephanensis* in alpine, temperate and tropical habitats. Where the average monthly temperature ranged from –7°C to +7°C in an alpine habitat at 2300 m altitude, almost only psychrotolerant strains were present in soil samples (Figure 46.13). Where the average monthly temperature varied from –4°C to +13°C in an alpine habitat at 1350 m, or from 0°C to 20°C in the temperate habitats, species of both thermal groups were detected. In the tropical habitat, the average monthly temperature ranged from 24°C to 32°C. Laboratory tests showed that the psychrotolerants could grow at those high temperatures, but more slowly than the mesophiles, and that they could not compete with them. Presumably, it was for that reason that the population in the tropical habitat consisted of mesophiles only (Von Stetten *et al.*, 1999).

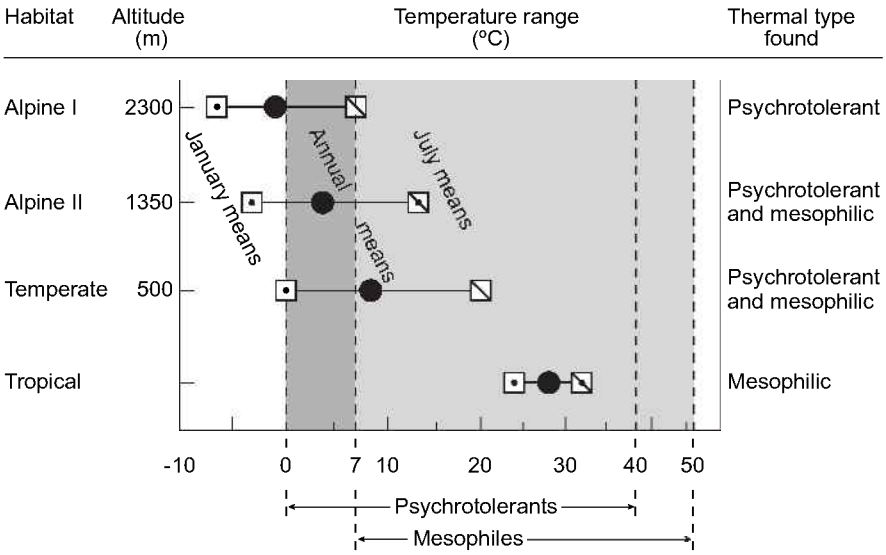


Figure 46.13 Influence of temperature range on the distribution of three species of the *Bacillus cereus* group: (i) *B. weihenstephanensis*, psychrotolerant; (ii) *B. cereus* and *B. thuringiensis*, mesophilic. (From Von Stetten *et al.*, 1999.) Soil samples were obtained from two alpine habitats (at 2300 and 1350 m) and from single temperate region and tropical habitats. The Cereus Group species present in them were identified and their growth rates were measured at five temperatures between 7°C and 40°C. The white areas of the chart indicate the temperatures, below 0°C and above 46°C, at which no substantial growth occurs. The darkest area marks the temperature range between 0°C and 7°C within which only psychrotolerant strains grow well. The upper horizontal arrow shows the temperature range, 0°C to 38°C, within which growth of psychrotolerants is possible. The lower horizontal arrows marks the temperature range of >7°C to 46°C within which mesophilic strains grow.

A 1 g sample of soil from a wood in Japan contained an estimated 4.4×10^4 spores of *B. thuringiensis*. The sample provided ten isolates of *B. thuringiensis*; all showed moderate larvicidal activity against *St. aegypti* and *Cx. pipiens*, but not against larvae of two lepidopteran species. Of the ten isolates, one was *B. t. kyushuensis*, six were *B. thuringiensis amagiensis*, and three were unrecognized. All showed a strong immunological relationship with the type strain of *B. t. kyushuensis* (Ishii and Ohba, 1993).

(b) Soil ecology of Cereus Group species

Soil has been defined as 'a complex and dynamic assemblage of components: rock particles of varying size, air, humus, minerals in solution in water, and organisms including bacteria, worms and arthropods; it is a medium that provides for plant growth' (Clugston, 2004). How often soil contains organic compounds in solution in water at a sufficient concentration to satisfy heterotrophic microorganisms is uncertain. Only very few reports of Cereus Group species in soil indicate whether they were in the rhizosphere, the soil immediately surrounding plant roots (see Subsection 46.5.11.d below), into which plants secrete a wide range of nutrients.

The ability of *B. cereus* ATCC 14579 to grow in the water-soluble constituents of soil was tested with a medium called 'soil-extracted solubilized organic matter' (prepared by suspending 100 g air-dried topsoil in 500 ml of MOPS (3-(*N*-morpholino)propanesulphonic acid) buffer and shaking for 1 h, followed by filtration, adjustment to pH 6.5, and sterilization by further filtration). The *B. cereus* strain germinated, grew to a stationary phase and sporulated in that medium, leading to the conclusion that it had 'behaved as a saprophyte' (Vilain *et al.*, 2006).

Properly, saprophytes are 'plants that obtain nutriment from dead or decaying matter'. Two other terms are more appropriate to micro-organisms. (i) Saprobes – organisms that thrive in water rich in organic matter. (ii) Saprotrophs – organisms (typically bacteria or fungi) that feed by secreting digestive enzymes into an organic substratum and absorbing the digestion products directly into their cells. The description of *B. cereus* as saprophytic passes from author to author with little or no supporting evidence. This bacterium survives and perpetuates itself in a variety of ways. It survives and perpetuates itself in mammal intestines, for example in human food-borne gastrointestinal infections (Section 46.5.10.b). Filamentous, spore-forming microbes isolated from the intestines of >25 species of soil insects and isopod crustaceans were identified as species of *Bacillus*, and sequencing the 16S rRNA gene in isolates from four host species showed them to be *B. cereus*. Margulis *et al.* (1998) surmised that *B. cereus* is a normal inhabitant of animal intestines, living symbiotically.

The near ubiquity of *B. thuringiensis* in soil led Martin and Travers (1989) to question whether it exists in soil independently of its growth cycles in insects or whether its association with certain insects is obligatory. They noted that *B. thuringiensis* is not pathogenic to many insect larvae that live in soil, but is pathogenic to some larvae that live or feed above ground or in water.

Findings described here and in other sections lead to a number of conclusions, mostly provisional. *Bacillus anthracis* is an obligate pathogen of mammals; it does not multiply in soil but its spores can survive for long periods in soil. *Bacillus cereus* (*sensu stricto*) may be facultatively pathogenic (Section 46.5.10.b); slender evidence suggests that it may otherwise exist as a saprobe in soil. A case can be made for *B. thuringiensis* (*s.s.*) being obligatorily pathogenic in insect larvae, but able to survive as spores in soil. Biosynthesis of the large amount of protein in the crystalline parasporal bodies produces a substantial drain on individual bacteria, so the δ-endotoxins must provide a benefit, which presumably is the nutriment available in the carcasses of poisoned hosts. Strains of *B. cereus* and *B. thuringiensis* that acquired virulence genes from *B. anthracis*, and that in so doing became genomically distinct, possibly perpetuated themselves as pathogens of mammals (Section 46.5.1.b).

(d) Cereus Group species in the rhizosphere

Certain Cereus Group species have been shown to thrive in the rhizosphere, a habitat defined as 'the soil immediately surrounding plant roots that is influenced structurally or biologically by the presence of those roots'. The root-surface component of the rhizosphere is called the rhizoplane (Lincoln *et al.*, 1998). Many microorganisms thrive in the rhizosphere that are not found in high density in soil outside it. The Cereus Group species *Bacillus mycoides* has been isolated from the outer surface of roots of the Douglas fir (*Pseudotsuga menzeisii*) (Petersen *et al.*, 1995). In tea-growing areas in India, the long life of the tea bushes was found to favour populations of bacteria and fungi in the rhizosphere. *Bacillus mycoides* and *B. subtilis* formed a major part of the bacterial population, their endospore-producing capability giving them advantage over other bacteria. Even in the winter months, when the soil temperature was 0–5°C, the *B. mycoides* population reached 10^7 cells g^{-1} rhizosphere soil, but in the laboratory they did not grow when kept below 14°C. *Bacillus mycoides* is present also at the rhizoplane; plant roots placed on agar gel were surrounded by rhizoidal growth of *B. mycoides* within 48 h (Pandey and Palni, 1997; Pandey *et al.*, 2001).

Little is known of the capability for growth of *B. anthracis* outside its mammalian hosts. In an artificial system of grass (*Festuca arundinacea*) growing in potting compost, spores of *B. anthracis* germinated on and around the roots, and grew in characteristic long filaments. When two strains of *B. anthracis* were inoculated into such a habitat, the tetracycline resistance plasmid pBC16 was transferred from a donor to a recipient strain. Saile and Koehler (2006) concluded that *B. anthracis* can perpetuate itself outside its mammalian hosts.

Bacillus t. israelensis was isolated from the rhizosphere of a *Ficus doliaria* tree in a National Forest Preserve in Bahia, Brazil, where contamination by commercial microbial insecticides was considered impossible (Rabinovitch *et al.*, 1999). Most investigations of *B. thuringiensis* have focused on exudation of δ-endotoxins into the rhizosphere by genetically modified crop plants. Under maize crops that expressed Cry1Ab, the amount of the toxin in bulk soil was always lower than that in the rhizosphere soil, but even so immunoreactive Cry1Ab was detected in bulk soil, at 0.21 ng g^{-1}, 7 months after harvesting (Baumgarte and Tebbe, 2005). δ-Endotoxins from *B. t. kurstaki* and *B. thuringiensis tenebrionis* adhered to clay-sized particles separated from loam, and the endotoxin-bearing particles were toxic to lepidopteran or coleopteran larvae when they were added to artificial diets. The persistence of extruded toxins in soil was ascribed to their adherence to clay particles, supposedly by both adsorption and binding (Tapp and Stotzky, 1995).

(e) Persistence in soil

How long do the spores of Cereus Group species survive in soil, and are these species capable of vegetative growth in soil?

Because of the potential of airborne spores of *B. anthracis* as a biological weapon, the persistence of the spores in soil has attracted particular interest. In 1942 and 1943, clouds of spores were released and carried by wind over the 211 ha Gruinard Island, off the north-west coast of Scotland, causing the infection and death of sheep pastured downwind of the release site. Annual soil sampling from 1946 to 1949 showed that, although spore density was gradually declining, measurable contamination was likely to persist for many years. In 1979, no spores were recovered from the pasture where sheep had died; however, spores were recovered, mostly in the top 10 cm of the soil, to the north and east of the release site owing to wind-assisted dispersal, and also to the south of it, largely owing to drainage (Manchee *et al.*, 1994). Death of the host enables massive vegetative growth of *B. anthracis* in the carcass, whereas multiplication of the pathogen in soil is thought to be very limited (Pepper and Gentry, 2002).

In one study, spores and crystals from a commercial preparation of *B. thuringiensis* serotype H3a3b-2 were added to soil at a number of sites in the field. During the first 2 weeks after their addition, the spore count decreased by ~1 log, but thereafter it remained constant for at least 8 months. Survival of the spores was thought to result from their inability to germinate in soil; spores removed from soil and placed on culture plates in the laboratory germinated. Spores that were produced in soil after the addition of vegetative cells survived for only a short time. Over an 8-week period there was no change in the appearance of the toxin crystals, but their toxicity was not tested (Petras and Casida, 1985).

To study the persistence of *B. thuringiensis* in soil, spores of *B. t. kurstaki* (DMU67R), a spontaneous mutant and identifiable marker, were sprayed on to soil, resulting in a concentration of 1.2×10^4 CFU g^{-1} soil to a depth of 2 cm. After 1 year that titre had declined to 2.3×10^3 CFU g^{-1} soil, 77% of the total remaining being in the top 2 cm of soil. Linear regression analysis indicated a half-life in soil of 120 days (Pedersen *et al.*, 1995).

(f) Occurrence on foliage

Bacillus cereus and *B. thuringiensis* are among the many species of bacteria that have been isolated from leaf surfaces, the so-called 'phylloplane'.

In North America, the numbers of bacteria were counted on leaves from a wide variety of shrubs and trees; the leaves had been picked 2.0–2.5 m above the ground to minimize soil splash, and inside the outer leaf canopy to minimize wind-borne contamination. *Bacillus thuringiensis* was isolated from the leaf surfaces of species of six genera. Where it occurred at densities of >10 bacteria per cm^2 (the cut-off for casual isolates) *B. thuringiensis* was recognized as a phylloplane epiphyte, i.e. part of the common leaf microflora. Among SDS extracts of bacteria from leaf samples (n = 81), 63% reacted to antibodies against anti-

lepidopteran toxins, 16% to antibodies against anti-coleopteran toxins and 2% to antibodies against anti-dipteran toxins. Consistent with that finding, SDS-PAGE analysis identified the subspecies *B. t. kurstaki*, *B. t. tenebrionis* and *B. t. israelensis* (Smith and Couche, 1991).

In the Netherlands, samples of grass foliage were collected on four occasions, during March through to May, from a pasture where patches without grass foliage indicated damage by larvae of *Tipula* spp. (Diptera, Tipulidae). These larvae live in the soil but emerge at night to feed on grass foliage. Among 32 isolates of *B. thuringiensis* from the grass foliage, 75% were of *B. t. israelensis*, and 84% of those were pathogenic to *St. aegypti* larvae. Four isolates of *B. t. israelensis* were highly or moderately pathogenic to larvae of *Tipula oleracea*. Six other subspecies or H serovars of *B. thuringiensis* were found, but none of the isolates was pathogenic to larvae of *St. aegypti* or to *Pieris brassicae* (Lepidoptera). These findings were consistent with the hypothesis that the distribution of populations of *B. thuringiensis* subspecies correlates with the taxa of the insects feeding on the leaves and roots (Damgaard *et al.*, 1998). Previously, Damgaard *et al.* (1997) had surmised that the principal insect species on a phylloplane may support an enzootic population of a *B. thuringiensis* subspecies to which it is susceptible.

(g) *Occurrence in aquatic habitats*

Since the discovery of *B. t. israelensis* in desert pools (described in Subsection 46.5.11.h below), only a few investigations have been undertaken of the occurrence of *B. thuringiensis* in aquatic habitats. In Kyushu, Japan, *B. thuringiensis* was isolated from 49.5% of samples from running-water habitats (rivers, streams, ditches) and still-water habitats (ponds) (n = 107), representing only 4.4% of Cereus Group isolates (n = 4414). Its density in the water samples ranged from 0 to 7.78 CFU ml^{-1} (mean 0.45 CFU ml^{-1}). The *B. thuringiensis* isolates (n = 195) were assigned to 26 different H serotypes. The largest group (n = 29) was of isolates identified as *B. t. israelensis/B. thuringiensis malaysiensis*, all of which had been collected in the same locality. Of the *B. thuringiensis* isolates, 26.7% were toxic to *Cx. pipiens* larvae (Ichimatsu *et al.*, 2000).

(h) *The discovery of* Bacillus thuringiensis israelensis

Bacillus thuringiensis israelensis is the subspecies of highest toxicity to mosquito larvae, and can be formulated to produce an effective bioinsecticide. It has been recorded in water, in soil and on foliage, as noted above. During 1975–1976 a survey was undertaken in Israel for new biocontrol agents active against mosquitoes, in which the investigators screened bacterial clones isolated from soil samples taken from known mosquito larval habitats. Ten bacterial isolates toxic to *Cx. pipiens* larvae were obtained, but only one (ONR 60A) possessed substantial activity. Strain 60A was collected in the north-western region of the Negev Desert, from a shallow, stagnant floodwater pool, the surface of which was thickly covered with dead and dying *Cx. pipiens* larvae (Figure 46.14). A sample collected from the edge of the pool contained dead larvae, water and silty mud. Clones of bacteria from this sample (which constituted strain 60A) showed larvicidal activity against species of *Culex*, *Stegomyia*, *Uranotaenia* and *Anopheles* (Goldberg and Margalit, 1977; Margalit and Dean, 1985).

When examined at the Institut Pasteur in Paris, clones of strain 60A were found to be antigenically distinct from the 13 known flagellar serotypes of *B. thuringiensis*, and to differ in some biochemical characteristics, therefore the strain was designated as serotype H14 and named *Bacillus thuringiensis* var. *israelensis* (de Barjac, 1978).

Later, samples were taken from larval habitats in northern Israel and the Negev Desert in southern Israel, first when water and mosquito larvae were present and again later when the sites were dry. The samples were heat treated to kill non-spore-forming organisms and vegetative cells. No pathogenic species of bacteria were recovered from the upper layers of water in the pools. However, 130 samples obtained from the edges of drying pools that contained mosquito larvae, or from soil at the

Figure 46.14 Photographed in 1984, a drying stagnant pool located at the site where *Bacillus thuringiensis israelensis* was first recovered in 1976, near Kibbutz Zeelim in the Nahal Besor Desert river basin, in the north-western Negev Desert of Israel. At that earlier date the pool size was 15 × 60 m, with a maximum depth of 0.3 m, a salinity of 900 mg Cl^{-} l^{-1}, and a heavy load of decomposing organic matter. The narrow shadow thrown across the pool was from a person standing at the right (From Margalit, 1990.)

bottom of dried pools, yielded several hundred isolates of spore-forming bacteria, including some that were toxic to *Culex* and *Stegomyia* larvae. By flagellar-agglutination assay, certain of the isolates were shown to be *B. t. israelensis* (Brownbridge and Margalit, 1986; Margalit, 1990).

The north-west region of the Negev Desert receives erratic winter rainfall. Most of the area is covered with fine grained loess (a deposit of wind-blown sediment) which is transported further by wind or by winter streams descending from the Negev hills. During the first winter rains, the surface grains swell and coalesce into a hard crust that is impenetrable to the seepage of additional rainwater into the subsoil. Rainwater then collects on the surface, and flash floods tear open deep gullies, leaving temporary pools of 1–200 m^2 and 0.5–4 m depth which may last until early summer. Eventually, the pools dry out and the loess surface crumbles, when fine particles can be transported in dust storms, carrying with them microorganisms and their spores. This may explain the greater prevalence of spore-forming entomopathogenic bacilli, including *B. thuringiensis* and *L. sphaericus*, in the Negev area (Dimentman and Margalit, 1981; Margalit, 1990).

(i) Epizootiology

Damgaard (2000) commented that epizootics of *B. thuringiensis* are rarely encountered in nature, and that only a few well-described cases are known. There is no clear-cut point of distinction between enzootics and epizootics, but it is likely that enzootics of *B. thuringiensis* would not be detectable by observation alone. One of the few reported cases of an epizootic caused by *B. thuringiensis* (and noted in Subsection 46.5.11.h above) had resulted in 'a thick carpet' of dead and dying *Cx. pipiens* larvae lying at the surface of a pool in the Negev Desert. An 'apparent epizootic' of *B. thuringiensis* infecting larvae of *Helicoverpa armigera* (Lepidoptera) was reported from a wheat field in Kenya (Brownbridge and Onyango, 1992).

Epizootics of *B. thuringiensis* have been reported more often from insect-rearing facilities, for example, at an early date from silkworm-rearing facilities (Ishiwata, 1901; cited by Damgaard, 2000), and more recently from mass cultures of the cotton bollworm (*Pectinophora gossypiella*) and the European sunflower moth (*Homoesoma nebulella*) (Dulmage, 1970; Itoua-Apoyolo *et al.*, 1995). Most strains of *B. thuringiensis* that have been used commercially were first isolated from diseased insects, probably obtained during epizootics, e.g. *B. t. kurstaki* from *Ephestia kühniella* (Lepidoptera), *B. t. israelensis* from *Culex pipiens* (Diptera), and *B. t. tenebrionis* from *Tenebrio molitor* (Coleoptera) (Damgaard, 2000).

46.6 *LYSINIBACILLUS SPHAERICUS*

The bacterium *Bacillus sphaericus* was isolated from moribund larvae of *Culiseta incidens* collected in California in 1965. During the following decades it was a subject of extensive research, particularly into the toxins that it produced and the mechanisms of their toxicity, but it was not until 1996 that, following extensive strain selection, it was shown to have potential as an effective larval biocide under field conditions. Now, water-dispersible, granular formulations of the crystalline Bin protoxin, which is produced in large amounts during sporulation, are the most effective control agents for larvae of *Cx. pipiens* and *Cx. quinquefasciatus*. The formulated toxin is persistent following dispersal in natural water bodies, and even in the highly polluted water of pit latrines. Following taxonomic investigations, in 2007, *B. sphaericus* was transferred to the new genus *Lysinibacillus*. This section is devoted to descriptions of the cell-growth cycle and sporulation of *L. sphaericus*, the range of toxins that it produces, their modes of action and their pathogenic actions on *Culex* larvae.

46.6.1 Systematics

(a) Species identity and nomenclature

The species of bacterium that for over a century was known as *Bacillus sphaericus* Meyer and Neide is highly heterogeneous. Phenotypically, it has rod-shaped vegetative cells and produces spherical spores, the reason for its specific name. It is one of a small number of closely related species that produced spherical spores and that are aerobic and mesophilic. They are unable to use sugars as sources of energy or of carbon for growth, but instead channel acetate and certain amino acids into the tricarboxylic acid cycle. The species with spherical spores respond negatively to many traditional taxonomic tests, such as the production of acid from sugars or the hydrolysis of starch, so in the absence of further diagnostic characters all aerobic, spherical-spored bacilli were classified as *B. sphaericus* (Priest and Dewar, 2000).

A phylogenetic analysis of 16S rDNA sequences from 58 strains of *Bacillus sphaericus* and strains of closely related taxa segregated them into seven clusters, which corresponded with groupings based on phenotypic analysis. Cluster 2 represented *Bacillus fusiformis* and included its type strain, ATCC 7055. Cluster 3 represented *B. sphaericus s.s.* and included its type strain, ATCC 14577. Cluster 1, which included insect-associated and mosquito-larvicidal strains, was closely linked to cluster 2. The remaining four clusters represented unnamed taxa (Nakamura, 2000). In a later phylogenetic study, clusters 6 and 7 were differentiated from clusters 1 to 5 by low DNA relatedness and differences in fatty acid composition, and they were taken to represent two novel species, *Bacillus pycnus* and *Bacillus neidei*, respectively (Nakamura *et al.*, 2002).

Later, characterization of three strains of a rod-shaped, spore-forming, motile, Gram-positive and boron-tolerant bacterium isolated from soil led to the proposal that they be treated as a new genus, *Lysinibacillus* Ahmed 2007, and a new species *Lysinibacillus boronitolerans*. Comparative analysis of 16S rDNA sequences revealed a close relationship between that new species and *B. sphaericus* s.s. and *B. fusiformis*, and it was proposed that the latter two species be transferred to the genus *Lysinibacillus* as *L. sphaericus* and *L. fusiformis*, respectively (Ahmed *et al.*, 2007). The generic name *Lysinibacillus* Ahmed 2007, with the names of four species, including *L. boronitolerans, L. sphaericus* and *L. fusiformis*, are published in the *List of Prokaryotic Names with Standing in Nomenclature* (Euzéby, 2011), and are used in this volume.

(b) Pathogenic forms

Early studies showed that *Lysinibacillus sphaericus s.l.* occurs worldwide, in soil and in aquatic habitats, supposedly as a saprobic organism which under adverse conditions produces spores. During sporulation, some strains formed a crystalline, proteinaceous, parasporal body within an elongated exosporium, and produced toxins that were pathogenic to mosquito larvae. The first isolate with mosquito-larvicidal activity was obtained from moribund larvae of *Culiseta incidens* found in freshwater rock holes in California (Kellen *et al.*, 1965). Later, many other isolates of *L. sphaericus* that were pathogenic or toxic to mosquitoes were obtained from different parts of the world. In an investigation in Israel, samples of mud, soil and moribund or dead larvae were taken from water-filled pools, and soil samples were collected again when the pools had dried. Of 19 *L. sphaericus* isolates bioassayed against fourth-instar *Cx. pipiens* larvae, five proved highly pathogenic and four moderately pathogenic (Brownbridge and Margalit, 1986, 1987).

Early attempts to classify the strains of *L. sphaericus* grouped them according to a variety of criteria (Table 46.9): (i) H serotypes based on flagellar antigens; (ii) DNA homologies (groups I to V); (iii) susceptibility to different bacteriophages; and (iv) biochemical characteristics, including fatty acid profile. Strains that showed high or moderate mosquito- larvicidal activity were of DNA group IIA and possessed genes for binary toxin (Krych *et al.*, 1980; Yousten, 1984; de Barjac *et al.*, 1985; Charles *et al.*, 1996). The more recent analysis of 16S rDNA sequences (noted above), which separated the strains of *L. sphaericus* and closely related species into seven clusters, showed that the strains pathogenic for mosquito larvae were not strains of *L. sphaericus sensu stricto*, but were members of a different cluster, more closely related to *L. fusiformis* (Nakamura, 2000).

Populations of *L. sphaericus* are highly clonal. For example, strains assigned to the clone associated with serotype H5a5b have been isolated in all parts of the world; they all showed the same chromosome structure and, where examined, had identical crystal protein structural genes (Priest and Dewar, 2000).

Table 46.9 Comparison of the characteristics of nine strains of *Lysinibacillus sphaericus s.l.* isolated from locations in eight different countries. Strain ATCC 14577 is the type strain of *L. sphaericus*. (From Baumann *et al.*, 1991; Charles *et al.*, 1996; and Liu *et al.*, 1996.)

Flagellar antigen	*Strain*	*Phage group*	*DNA group*	*Mosquito larvicidal activity*	*Binary toxin genes*	*Mtx genes*		
						Mtx1	*Mtx2*	*Mtx3*
H 1a	Kellen Q	1	IIA	Low	−	+	+	+
H 2a2b	SSII-1	2	IIA	Moderate	−	+	+	+
H 3	LP1-G	8	IIA	Moderate	+	−	?	?
H 4	ATCC 14577	*	I	Absent	−	?	?	?
H 5a5b	1593	3	IIA	High	+	+	+	?
H 5a5b	1691	3	IIA	High	+	+	+	+
H 6	IAB 59	3	?	High	+	+	+	+
H 9a9c	31	8	?	Low	−	+	+	+
H 25	2297	4	IIA	High	+	+	+	+
H 26a26b	2173 (ISPC5)	*	IIA	Moderate	−	−	−	?

Larvicidal activity was measured with fourth-instar *Culex pipiens* larvae, after 48 h exposure. Low activity, $LC_{50} \geq 10^{-3}$ of sporulated culture medium. Moderate activity, $LC_{50} \approx 10^{-4}$. High activity, $LC_{50} \leq 10^{-6}$. Contrary to the findings shown in this table, Thanabalu and Porter (1995) did not detect Mtx1 in strains Kellen Q, IAB 59 or 31.
+, present; −, absent; ?, no information available.
*, Not responding to any of the bacteriophages tested.

46.6.2 Cell growth and sporulation

Lysinibacillus sphaericus passes through the growth phases (lag – exponential – stationary) that are characteristic of species of *Bacillus* and *Clostridium* (Section 46.3) .When strain 2297 of *L. sphaericus* was grown in a liquid medium at 30 °C, the mean duration of the exponential phase was about 60 min, and sporulation was completed within 24 h of inoculation. Among cultures that had been inoculated simultaneously, the cells developed synchronously and passed synchronously through the stages of sporulation at the time course shown in Figure 46.15. A close correlation was observed also between cultures in the development of crystalline inclusions and their toxicity to mosquito larvae (Kalfon *et al.*, 1984).

A septum that would separate off one end of the cell first appeared during the time period t_1 and became fully formed during t_2 (Stage II of sporulation) (Figure 46.15). Shortly afterwards, electron micrographs revealed a growing parasporal body already showing a crystalline structure. The prespore and parasporal body were engulfed during the period t_3 (Stage III). By the start of period t_4 the prespore had an irregular oval shape, and the crystal-like parasporal inclusion, 0.5 μm wide, already had its typical shape and structure. Synthesis of the exosporium and spore coat began during the period t_5, and the two layers of the cortex were present during t_6, by which time a spore coat completely enveloped the forespore. During t_8 (Stage V), the cortex and spore coat became fully formed, and by t_9 the exosporium was completely formed. During t_{10}, the beginning of lysis was observed, and by t_{15} the sporangia were completely lysed and exosporia containing both spore and inclusion were released into the medium.

The toxicity of the culture medium to fourth-instar *Cx. pipiens* larvae was expressed in terms of dilutions of samples of the culture medium that produced an LC_{50} or LC_{90}. It varied with time after inoculation. By 24 h post-inoculation the LC_{50} had reached 1.09×10^{-7}, a level of toxicity $>10^3$-fold greater than that with vegetative cells at t_{-2} ($LC_{50} = 8.4 \times 10^{-3}$). The principal increase in toxicity, shown by decreases in LC_{50} and LC_{90}, occurred by t_4 (Kalfon *et al.*, 1984).

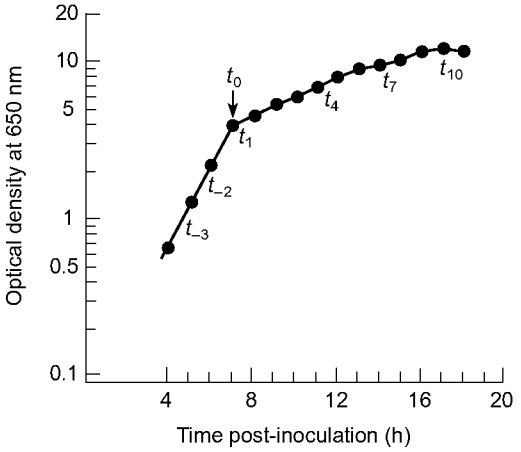


Figure 46.15 Partial growth curve of a culture of *Lysinibacillus sphaericus* (strain 2297) developing in a liquid medium at 30°C, showing the exponential and stationary phases. (After Kalfon *et al.*, 1984.) A 5 ml inoculum from a sporulated culture that had been heat shocked to synchronize cell growth was added to 100 ml of medium adjusted to pH 7.4; and incubated with shaking. The estimated time of start of sporulation (t_0) was determined graphically from the growth curve. The time periods t_2 to t_{10} (between points marked on the curve) correspond to the stages II to VII of sporulation described in Section 46.3.b.

46.6.3 Mosquitocidal (Mtx) toxins

Two types of toxin are known from the mosquito-larvicidal strains of *L. sphaericus*: (i) so-called mosquitocidal toxins, which appear to be associated with the bacterial cell membrane; and (ii) binary toxin (Bin), which is associated with the crystalline parasporal body. From the limited data presented in Table 46.9, which shows Bin-expressing genes to be present in strains of *L. sphaericus* with high mosquito-larvicidal activity, but absent from strains with low activity and most strains with moderate activity, it appears that Bin is responsible for most of the toxicity of strains that have high larvicidal activity.

Three mosquito-larvicidal toxins have been described, named Mtx1 (formerly Mtx), Mtx2 and Mtx3, which have molecular masses of 100.6, 31.8 and 35.8 kDa, respectively. The genes that encode them, *mtx1*, *mtx2* and *mtx3*, are widely distributed among mosquitocidal strains of *L. sphaericus*, being

present in both crystal-forming and non-crystal-forming strains. The genes *mtx1* and *mtx2* are expressed during the vegetative growth phase, as probably is *mtx3* also. The strains of *L. sphaericus* with low mosquito-larvicidal activity lack a parasporal body, as also does strain SSII-1, which shows moderate larvicidal activity. SSII-1 produces toxin predominantly during the vegetative phase of growth, before the onset of sporulation, so apparently Mtx proteins are responsible for its toxicity (Thanabalu *et al.*, 1991; Porter *et al.*, 1993; Charles *et al.*, 1996).

Mtx1 has a molecular mass of 100.6 kDa. In five strains of *L. sphaericus* that produced Mtx1, the protein was clearly detectable during the vegetative phase. In strain SSII-1, Mtx1 was associated with the soluble fraction of vegetative cells; it was undetectable in either soluble or pellet fractions of sporulated cells. Some strains of *L. sphaericus* produce both intracellular and extracellular proteases, and the disappearance of Mtx1 was ascribed to protease produced during sporulation. Incubation of Mtx1 with extracts of vegetative cells did not degrade it, but incubation with extracellular protease produced during sporulation led to its degradation within 16 h (Thanabalu and Porter, 1995).

Mtx1 is synthesized as a single chain of 870 amino acid residues. Primary processing results in the severance of 29 N-terminal residues (the putative signal sequence), yielding the 97 kDa holotoxin named $Mtx^{30–870}$. That can be cleaved into two parts by larval gut extract or chymotrypsin: (i) to an N-terminal, 27 kDa moiety, $Mtx^{30–264}$, which consists of four ricin-B-like domains that possess ADP-ribosyltransferase activity; and (ii) to a C-terminal, 70 kDa moiety, $Mtx^{265–870}$, the putative binding and translocation domain (Table 46.10A) (Thanabalu *et al.*, 1992; Schirmer *et al.*, 2002).

A study of the kinetics of the ADP-ribosyltransferase revealed that the products of proteolytic (chymotrypsin) cleavage of the holotoxin ($Mtx^{30–870}$) had much lower affinity for the substrate (K_m) than did the products of a cleaved 32 kDa N-terminal fragment ($Mtx^{30–308}$) construct (which included the 27 kDa $Mtx^{30–264}$ moiety described above) alone (Table 46.10B). Schirmer *et al.* (2002) postulated that the 70 kDa fragment remained non-covalently bound to the 27 kDa fragment, thereby inhibiting its ADP-ribosyl-

Table 46.10 Molecular and enzymatic characteristics of the mosquitocidal toxin Mtx1 of *Lysinibacillus sphaericus* strain SSII-1. (From the data of Thanabalu *et al.*, 1992; and Schirmer *et al.*, 2002.)

A. Mtx1 and its cleavage products

Mtx1 as synthesized: molecular mass 100.6 kDa; 870 amino acid residues. During primary processing, an N-terminal, putative signal sequence of 29 residues is removed, yielding the holotoxin.

Holotoxin = $Mtx^{30–870}$. Molecular mass ~97 kDa. The native form of the toxin, which consists of amino acid residues 30 to 870.

Chymotrypsin-cleavage products of the holotoxin:

$Mtx^{30–264}$: N-terminal, 27 kDa fragment; consists of four ricin-B-like domains that possess ADP-ribosyltransferase activity.

$Mtx^{265–870}$: C-terminal, 70 kDa C fragment; putative binding and translocation domain.

B. Kinetics of ADP-ribosyltransferase activity

*Mtx cleavage products**	K_m *(NAD)* μM	k_{cat} min^{-1}	$k_{cat}:K_m$ min^{-1}
$Mtx^{30–870}$ holotoxin	1300 ± 196	0.5 ± 0.3	0.0004
$Mtx^{30–308}$ alone	45 ± 7	2.5 ±1	0.06

*, Products of chymotrypsin cleavage of the holotoxin and of a 32 kDa N-terminal Mtx construct, $Mtx^{30–308}$ (which include $Mtx^{30–264}$).

ADP-ribosylation of soybean trypsin inhibitor was measured in the presence of [^{32}P]-NAD. Kinetic values were obtained from Lineweaver-Burk plot transformation of data. Data are given as means ± s.e. (n = 3).

transferase activity. Only denaturing procedures separated the two fragments. In the presence of an equimolar concentration of the 70 kDa moiety, the ADP-transferyl activity of the 27 kDa moiety was reduced by 93.2%.

Mtx1 holotoxin was lethal to larvae of *Cx. quinquefasciatus* and *St. aegypti*, whether proteolytically cleaved or not. The N-terminal or C-terminal regions alone were non-toxic to *Cx. quinquefasciatus* larvae; both regions were required for toxicity (Thanabalu *et al.*, 1991, 1993). A domain in the C-terminal region of Mtx1 was sufficient for cytotoxicity to cultured cells from *Cx. quinquefasciatus* and *St. aegypti*, but not to cells from *An. gambiae* (Thanabalu *et al.*, 1993). Transfection of HeLa cells with the 70 kDa $Mtx^{265-870}$ led to cytotoxic effects such as cell rounding and formation of a thread-like protrusion (Schirmer *et al.*, 2002).

Sequence analysis of the cloned *mtx1* gene from *L. sphaericus* strain 2297 revealed a single open reading frame encoding an 870-amino acid polypeptide. Expression of the gene in *E. coli* was low unless the putative leader sequence was deleted and the truncated gene was expressed as a fusion protein with glutathione S-transferase (GST-tMx1). Cells of *E. coli* that expressed GST-tMx1 were highly toxic to *Cx. quinquefasciatus* larvae, but less toxic to larvae of *Anopheles dirus* and *St. aegypti* (Promdonkoy *et al.*, 2004).

Mtx2 has a molecular mass of 31.8 kDa, and not a great deal is known about it. The gene *mtx2*, which encodes a protein of 292 amino acids, is present in a number of mosquitocidal strains (Table 46.9). The protein had an estimated LC_{50} to first-instar *Cx. quinquefasciatus* larvae of 320 ng ml^{-1}. Mtx2 shows no significant sequence homology with Mtx1 or with other known insecticidal toxins, except for Mtx3, but it shares homology with two proteins that are toxic to mammalian cells, namely the ε-toxin of *Clostridium perfringens* and the cytotoxin of *Pseudomonas aeruginosa*. Purified recombinant Mtx2 was toxic to *Cx. quinquefasciatus* larvae (Thanabalu and Porter, 1996).

Mtx3 has a molecular mass of 35.8 kDa. The gene *mtx3* is highly conserved and is widely distributed among larvicidal strains of *L. sphaericus* of both high and low toxicity. Circumstantial evidence suggests that *mtx3* is expressed during the vegetative phase. The deduced protein shares homologies with Mtx2, the ε-toxin of *Clostridium perfringens* and the cytotoxin of *Pseudomonas aeruginosa*. After transfection, recombinant *E. coli* was moderately toxic to *Cx. quinquefasciatus* larvae and weakly toxic to *St. aegypti* larvae (Liu *et al.*, 1996).

46.6.4 Binary toxin

Binary toxin (Bin), a heterodimer composed of two polypeptides – BinA and BinB – is the constituent matter of the crystalline parasporal bodies formed during sporulation. Parasporal bodies with a crystal lattice structure are produced by all strains of *L. sphaericus s.l.* that have high larvicidal activity, e.g. strains 1593 and 1691 (both of serotype H 5a5b and phage group 3) and strain 2297 (serotype H 25 and phage group 4), and by certain less pathogenic strains also (Table 46.9). Each bacterial cell contains a single parasporal body, which is not composed of separate inclusions and which is surrounded by an envelope. It is a paralleliped, i.e. each of its six external faces has the shape of a parallelogram, and opposite faces are identical and parallel. Both spore and parasporal body are formed within an elongated exosporium which keeps them together after cell lysis (Davidson and Yousten, 1990).

The binary toxin genes of *L. sphaericus* have been detected in chromosomal DNA (Liu *et al.*, 1993; Priest *et al.*, 1997). A 3 kbp *Hind*III DNA fragment coding for the binary toxin has two open reading frames (ORFs) separated by an intergenic region of 174–176 base pairs, so the genes are organized in a single transcriptional unit or operon. The two ORFs coded for proteins of 370 and 448 amino acids, with deduced molecular masses of 41.9 (BinA) and 51.4 kDa (BinB), respectively (Arapinis *et al.*, 1988; Berry *et al.*, 1989). Transcription starts just before the end of the phase of exponential growth and continues into the stationary phase. Using translational *lacZ* fusion to the promoter of the binary-toxin gene enabled expression of the

gene to be monitored as β-galactosidase activity. During batch culture, β-galactosidase activity first appeared when growth deviated from an exponential pattern, and synthesis continued into the stationary phase for a further 11 h before declining (Kalfon *et al.*, 1984; Baumann *et al.*, 1988; Ahmed *et al.*, 1995).

When genes of the 41.9 and 51.4 kDa proteins were cloned into expression vectors and introduced into separate cells of an acrystallogenic strain of *L. sphaericus*, transformants containing either gene expressed individually produced small non-crystalline inclusions which were not toxic to mosquito larvae. When both proteins were expressed together, typical wild-type crystals were produced and toxicity was restored (Broadwell *et al.*, 1990c). Similar results were obtained when binary-toxin genes were introduced into an acrystallogenic strain of *B. thuringiensis*, but in that case the crystalline inclusion bodies formed outside the exosporium (Nicolas *et al.*, 1993).

In a number of Bin-producing strains, the 41.9 and 51.4 kDa proteins are present both in the parasporal crystal and tightly bound within the structure of the cell wall. Lysozyme treatment renders these proteins soluble. Deposition of the proteins in the cell wall precedes parasporal crystal formation, and reaches a maximum in the late exponential growth phase. In the cell wall fraction, the 51.4 kDa protein slightly predominates over the 41.9; the reverse situation obtains in the parasporal crystal (Klein *et al.*, 2002).

SDS-PAGE analysis of NaOH extracts of *L. sphaericus* spores showed bands corresponding to those of BinB and BinA, and two other bands of 110 and 125 kDa. Dynamic light-scattering studies showed that a solution of the purified 110 and 125 kDa bands consisted almost entirely of a particle with a hydrodynamic radius of 5.6 ± 1.2 nm and a calculated molecular mass of 186 ± 38 kDa, indicating that the binary toxin can exist in solution as an oligomer comprising two copies each of BinB and BinA (Smith *et al.*, 2005).

When parasporal crystals ingested by *Cx. pipiens* larvae were exposed to high pH in the midgut, solubilization led to the appearance of proteins of 51.4 and 41.9 kDa. The 51.4 kDa protein became subject to proteolysis, which led to transient accumulation of a protein of 43.1 kDa (as determined by SDS-PAGE) (Table 46.11).

Table 46.11 Sequence of solubilization, activation and binding of the insecticidal crystal protein of *Lysinibacillus sphaericus* in the midgut of *Culex pipiens* larvae that have ingested *L. sphaericus* cells.

Event	*Identity of proteins*	*Properties of proteins*
L. sphaericus cells ingested, moved to midgut lumen	Binary toxin	Within crystalline parasporal bodies. Insoluble. Non-toxic
↓	↓ ↓	
High pH in midgut solubilizes crystal proteins		
↓	41.9 kDa + 51.4 kDa	Soluble. ?Toxic
Midgut proteases shorten the proteins at N and C terminals	↓ ↓	
↓	38.8 kDa + 43.1 kDa	Activated toxins. Toxic when present together
Activated toxins bind to receptor on cell membrane and enter cell with small vesicles		
↓		
Toxic effect is effected by unknown mechanism		

Consistent with that finding, larvae that had ingested amorphous inclusions containing only the 51.4 kDa protein accumulated a 43 kDa derivative. Within the midgut, the solubilized 41.9 kDa protein was slowly converted to a 39 kDa derivative, which lacked ten amino acids at the N-terminus and 17 amino acids at the C-terminus. The points of both deletions corresponded to potential chymotrypsin sites (Baumann *et al.*, 1985, 1991; Broadwell and Baumann, 1987; Broadwell *et al.*, 1990a).

Three different methods of amino-acid deletion from the N- and C-termini of the 51.4 and 41.9 kDa proteins have been used to find the minimum size of the active toxins. By means of site-directed mutagenesis, removal of 21 amino acids from the N-terminus and of 53 from the C-terminus of the 51.4 kDa toxin yielded a protein of a calculated 43.1 kDa. Similarly, removal of ten and 17 amino acids from the N- and C-termini, respectively, of the 41.9 kDa protein yielded a protein of a calculated 38.8 kDa. The modified 43.1 and 38.8 kDa proteins were chromatographically identical with the 43 and 39 kDa proteins that accumulated in the midgut of larvae that had ingested parasporal crystals. Neither protein alone was toxic to mosquito larvae, but the toxicity of the two together was greater than that of the crystal proteins (Broadwell *et al.*, 1990a,b; Clark and Baumann, 1990). It appears that the 43.1 and 38.8 kDa proteins are activated toxins.

The use of restriction enzymes to delete sections from the N- and C-termini of the gene encoding the 41.9 kDa protein showed that the active toxin had a minimum size of 338 amino acids (38.3 kDa). N-terminal deletions beyond the Ile 18 residue, and C-terminal deletions beyond the His 352 residue, resulted in loss of toxic activity and rapid degradation by host proteases. Larvicidal activity was obtained only in the presence of the 51.4 kDa protein (Šebo *et al.*, 1990). Studies with mutants in which amino acids had been deleted from the N- and C-termini showed that, with the 51.4 kDa protein, 34–39 amino acids were not required at the N-terminus, and 52–54 were not required at the C-terminus. With the 41.9 kDa protein, removal of seven amino acids from the C-terminus abolished toxicity, whereas at least 17 could be deleted from the N-terminus without loss of toxicity (Oei *et al.*, 1990).

The amino acid sequences of BinA and BinB from *L. sphaericus* are dissimilar from those of all other bacterial toxins, but they themselves share four segments of sequence similarity. They therefore constitute a separate family of insecticidal toxins. Comparison of the deduced amino acid sequences of BinA and BinB from strains 2297 and 2362 showed a difference of five amino acids between the 41.9 kDa proteins, and a difference of four amino acids between the two 51.4 kDa proteins (Davidson and Yousten, 1990). A more extensive comparison of the amino acid sequences in BinA and BinB from different strains of *L. sphaericus* showed them to be very highly conserved, in contrast to the insecticidal crystal proteins of *B. thuringiensis* (Berry *et al.*, 1989; Höfte and Whiteley, 1989).

46.6.5 Binding and pore formation by binary toxin

(a) Binding in vivo

To search for regions of binding and uptake of *L. sphaericus* toxin in the larval gut, larvae of *Cx. pipiens, St. aegypti* and four species of *Anopheles* were fed yeast suspension supplemented with fluorescein-labelled binary toxin. With *Cx. pipiens* larvae, the toxin bound to epithelial cells of the gastric caeca and posterior stomach, where it appeared to be internalized in small vesicles. With the *Anopheles* larvae also, the toxin bound to cells of the gastric caeca and posterior stomach, but in some individuals to cells of the anterior and central stomach also. Internalization was not observed, and the fluorescence leaked rapidly. The toxin did not bind to the midgut of *St. aegypti* larvae (Davidson, 1989). These different binding capabilities were consistent with the sensitivities to *L. sphaericus* toxin of the respective mosquito species (Table 46.12).

Further studies of binding were undertaken with *Cx. quinquefasciatus* larvae exposed to fluorescein-labelled BinA and BinB. When applied alone, BinB bound to the apical plasma membrane of cells in

Table 46.12 Toxicities of suspensions of *Lysinibacillus sphaericus* cells to the larvae of three mosquito species. The results are grouped according to serotype (flagellar antigen), and recorded as LC_{50} expressed as the dilution factor. A total of 151 strains, of six H-serotypes, were bioassayed. (From de Barjac, 1990.)

		LC_{50} (*mean*)		
Flagellar antigen	*No. of strains tested*	Culex pipiens	Anopheles stephensi	Stegomyia aegypti
H 5a5b	112	2.5×10^6	1.6×10^5	9.8×10^4
H 6	12	3.8×10^6	2.2×10^5	–
H 25	11	6.6×10^6	3.0×10^4	–
H 26a26b	5	3.5×10^4	–	–
H 2a2b	9	4.9×10^3	–	–
H 1a	2	1.0×10^2	–	–

Toxicities were measured after 48 h exposure of the larvae. The LC_{50} measurements indicate the extent of dilution of samples from the final whole culture. Results expressed as '–' indicate that LC_{50} values could not be determined because of low toxicities at 102-fold dilution.

the gastric caeca and posterior stomach, showing the same regional binding as the binary toxin, but it appeared not to be internalized. BinA bound to the apical membrane of cells throughout the midgut when applied alone, and also appeared not to be internalized. When both proteins were present, the binding of BinA showed the same regional distribution as BinB, i.e. to the gastric caeca and posterior stomach, and both proteins appeared to be internalized as small vesicles. These binding patterns suggested that BinB is the primary binding component of binary toxin, that it mediates the regional binding and internalization of BinA, and that a BinB–BinA complex, or possibly a BinB–BinA–receptor complex, is internalized. Deletion of amino acids from the amino- or carboxy-terminals of BinB revealed that at least a part of the amino-terminal region is required for binding to the larval gut, while at least a part of the carboxy-terminal region interacts with BinA. Toxicity was strongly correlated with internalization of the toxin (Oei *et al.*, 1992).

The postulated receptor for binary toxin in *Cx. pipiens* was named Cpm1 for the reason explained in Subsection 46.6.5.b below, and that in *Cx. quinquefasciatus* was named Cqm1. Events induced in target cells by binary toxin after it had bound to Cpm1 were investigated in cells from the mammalian cell line MDCK that had been transfected with *cpm1* DNA. Normal MDCK cells do not possess Cpm1 in their plasma membrane. In clonal lines that were recovered after the transfection, Cpm1 was shown to be a 67 kDa α-glycosidase anchored to the plasma membrane by a glycosylphosphatidylinositol moiety. [^{125}I]-Bin bound with high affinity to Cpm1/MDCK membrane, the dissociation constant (K_d) of 5.4 nM being very close to the K_d value for the brush border membrane fraction prepared from larval midgut cells. That suggested that Cpm1 had the same conformation on both cell surfaces. Immunofluorescence studies showed Cpm1 to be located on the apical side of polarized MDCK cells (Pauchet *et al.*, 2005).

(b) *Binding* in vitro

The presence of receptors on microvilli of the midgut epithelial cells was demonstrated by *in vitro* binding assays using brush border membrane preparations and [^{125}I]-labelled binary toxin. Specific, high-affinity binding of the toxin was mediated by a receptor with a unique binding site. Binding to membrane fractions from *Cx. quinquefasciatus* larvae indicated the presence of a single class of *L. sphaericus* binary-toxin receptor. The

dissociation constant (K_d) was 11–20 nM, and the maximum binding capacity was 7–8 pmol mg^{-1} of membrane protein (Nielsen-LeRoux and Charles, 1992; Nielsen-LeRoux *et al.*, 1995).

After solubilization of brush border membranes isolated from *Cx. pipiens* larvae, activated binary toxin bound to a 60 kDa protein, considered to be the receptor, which was anchored to the midgut membrane via a glycosylphosphatidylinositol anchor. Its peptide sequences were similar to those of members of the *a*-amylase family (Silva-Filha *et al.*, 1999). Later, cDNA corresponding to a 60 kDa protein isolated from midgut brush border membranes of *Cx. pipiens* larvae was cloned and characterized, when the deduced amino acid sequence showed 39–43% identity with insect maltases. For that reason, the protein was named Cpm1 – for '*Cx. pipiens* maltase 1'. Recombinant Cpm1 bound to Bin toxin and had significant *a*-glucosidase activity. Darboux *et al.* (2001) concluded that Cpm1 is an *a*-glucosidase that is expressed in *Culex* midgut, where it constitutes the receptor for Bin toxin.

Experiments with fractions from *An. gambiae* larvae showed binary toxin binding to a single class of receptor on brush border membrane fractions. The dissociation constant was 30 nm, and the maximum binding capacity 5 pmol mg^{-1}. Both BinA and BinB bound specifically, seeming to play an equivalent role in the binding, and it was concluded that binary toxin can bind to the *An. gambiae* receptor via either the BinA or BinB component. These proteins showed a slightly lower affinity for membrane fractions from *An. stephensi* (Silva-Filha *et al.*, 1997; Charles *et al.*, 1997). No significant binding could be detected of binary toxin to brush border membrane from *St. aegypti* larvae (Nielsen-LeRoux and Charles, 1992).

The binding characteristics of the BinA and BinB components of binary toxin were compared by adding [I^{125}]-labelled toxins singly or in combination with brush border membrane fractions. With membrane fractions from *Cx. pipiens* larvae, and in the absence of competitors, *c.* 3.3 pmol binary toxin bound per mg. BinB competed with binary toxin for the binding site, showing a similar affinity for it. BinA bound non-specifically until BinB was added. Binding was maximal when those two proteins were in an equimolar ratio (Charles *et al.*, 1997). Competition experiments showed that Bin1 and Bin2 share the same binding site in midgut brush border membranes from *Cx. pipiens* larvae. Their binding abilities were identical (Silva-Filha *et al.*, 2004). With *An. gambiae* larvae, *c.* 2 pmol binary toxin bound per mg membrane fraction. BinA bound more strongly than BinB, and the presence of either BinA or BinB enhanced the binding of the other component, so their actions are synergistic. Again, binding was maximal when the ratio was equimolar (Charles *et al.*, 1997).

To gain insight into the interactions of BinA and BinB with membranes, the interactions were examined in aqueous solution, singly or together and in the presence of lipid bilayers, using a form of infrared spectroscopy that is sensitive to changes in the secondary structure of proteins. BinA and BinB interacted when together in aqueous solution, forming a single *β*-structure, and it is in that state that they normally bind to a membrane. Once that *β*-structure had bound to a lipid bilayer, both moieties underwent conformational changes that affected their tertiary and secondary structures. The fact that different changes in conformation were seen after either BinA or BinB had bound individually to a lipid bilayer showed that the proper folding of each depends on the presence of the complementary subunit (Boonserm, *et al.*, 2006b).

A 19-nucleotide deletion in the *cqm1* allele was associated with the resistance of colonized *Cx. quinquefasciatus* larvae to a spore crystal preparation from *L. sphaericus*. The colony with the mutant allele, named $cqm1_{REC}$, had originated in the city of Recife in Pernambuco State, Brazil. In areas of Pernambuco State without a history of treatment with the *L. sphaericus* biocide, the $cqm1_{REC}$ resistance allele was detected only at very low frequencies, e.g. 0.0029 at Fazenda Nova and 0.0061 at Peizinhos. In contrast, in the treated area of Água Fri, the frequency was 0.053 (de Melo Chalegre *et al.*, 2009).

(c) Pore formation

The ability of binary toxin to permeabilize membranes was examined with receptor-free, unilamellar phospholipid vesicles loaded with calcein, the release of which could be recorded as an increase in fluorescence. Leakage of calcein from the vesicles occurred after exposure to binary toxin, the rate of leakage being highest at pHs between 9 and 10. From the hydrated Stokes radius of calcein, it was estimated that pores of at least 1.5 nm diameter formed in the vesicle walls. Presented alone, BinA permeabilized the vesicles, but only at high concentration and on long exposure. In contrast, presented alone, BinB was barely active but it enhanced the action of BinA (Schwartz *et al.*, 2001).

To obtain direct evidence of ion channel formation, planar lipid bilayers were exposed to BinA or Bin B, and step changes in current flow were recorded during the application of test voltages. Channel conductances ranged between 25 and 250 pS, but most current steps corresponded to conductances of 70–130 pS. BinA, when applied alone, formed voltage-dependent channels with long open times and a high open probability. BinB, which inserted less easily, formed smaller channels with shorter mean open times. Channels that formed from a 1:1 mixture of the two components displayed BinA-like activity (Schwartz *et al.*, 2001).

Postulating that those results reflected a detergent-like action, Boonserm *et al.* (2006b) measured the effects of BinA and BinB when introduced into the aqueous phase below a lipid bilayer in a Langmuir trough. BinA had no effect, but BinB inserted into the membrane. Introduction of BinA and BinB into the aqueous phase caused the same effect as BinB alone, but with different kinetics (Figure 46.16). The investigators concluded that BinA and BinB interact in solution and undergo a conformational change before binding to a specific or non-specific receptor, and that only BinB is involved in membrane insertion. These differences in the actions of BinA and BinB were consistent with their suggested roles *in vivo*, i.e. that BinB is the primary binding component of binary toxin, that it mediates the regional binding and internalization of BinA, and that a BinB–BinA complex is internalized (Section 46.6.5.a).

Electrophysiological recordings from patch-clamped MDCK cells showed that, after interacting with Cpm1, Bin modified the permeability of the plasma membrane of Cpm1-expressing MDCK cells, presumably by opening pores. If transfected MDCK cells were exposed to 25 nM Bin, small intracytoplasmic vacuoles appeared after 1 h and increased in size with increasing treatment

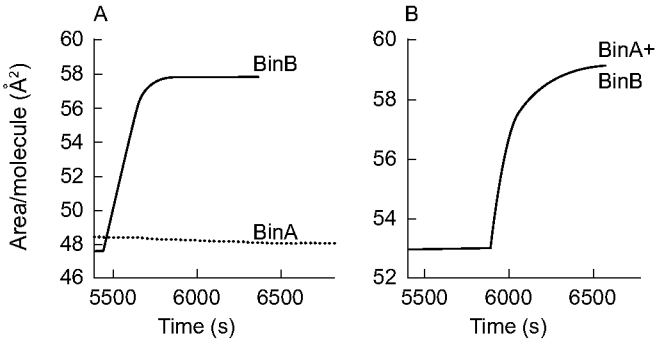


Figure 46.16 Insertion profiles of BinA and BinB from *Lysinibacillus sphaericus* in monolayers of dimyristoylphosphocholine (DMPC). **A**, BinA and BinB in separate assays. **B**, An equimolar mixture of BinA and BinB. (After Boonserm *et al.*, 2006b.) Interaction of BinA and BinB with the monolayers was monitored by a constant surface-pressure assay, using a Langmuir-Blodgett trough in which a solution of DMPC in chloroform was spread on the surface of carbonate buffer, pH 10. When a monolayer had formed it was compressed to a surface pressure of 25 mN m^{-1}, and a 3–5 nmol sample of the test protein was injected into the subphase. 1Å = 10^{-10} m; 10^{-1} nm.

duration. Pore formation and vacuolization did not lead to a breakdown of cell membrane (Pauchet *et al.*, 2005).

46.6.6 Toxicities

(a) Bioassays

de Barjac (1990) recommended that bioassays be carried out on early fourth-instar larvae of *Culex pipiens* or *Cx. quinquefasciatus*, the most sensitive species, scoring death rates after 48 or 72 h. Broadwell *et al.* (1990a) commented that there can be no standard bioassay for toxicity to mosquito larvae, because recorded LC_{50} values vary widely owing to the many variables.

Toxins have been prepared for bioassay in a variety of ways. Some investigators have taken samples from *L. sphaericus* cultures, diluting them and expressing LC_{50} and LC_{90} values in terms of the extent of dilution. Other investigators have used lyophilized cells, or inclusion body protein, or crystal protein, expressing toxicity as ng (dry weight) ml^{-1} needed to kill 50% of *Culex* larvae (LC_{50}). Assays of culture samples or of whole cells provide no information on the relative contribution to toxicity of the different toxins. Extracted parasporal bodies or crystal protein are likely to contain only Bin toxin. A bioassay of *L. sphaericus* strain 1593 crystal protein against *Cx. pipiens* larvae gave an LC_{50} of 2.3 ng protein ml^{-1} (Yuan *et al.*, 2001).

Among the mosquitoes that have been tested for susceptibility to *L. sphaericus*, *Culex pipiens* and *Cx. quinquefasciatus* were the most susceptible. Species of *Anopheles* varied from somewhat less to much less susceptible than the two *Culex* species. *Stegomyia aegypti* was relatively non-susceptible (Table 46.12). In addition, species of *Psorophora*, *Mansonia* and *Ochlerotatus* have proved to be susceptible (Lacey and Singer, 1982; Thiéry and de Barjac, 1989; Davidson and Yousten, 1990; Rodrigues *et al.*, 1998). A strain of *L. sphaericus* isolated from *Simulium damnosum* in Nigeria was pathogenic to *Cx. pipiens* larvae (Charles *et al.*, 1996), but most strains known to be pathogenic to mosquito larvae do not affect simuliid larvae. Information on the relative toxicities of different strains of *L. sphaericus* to mosquito larvae is of great commercial interest, e.g. the finding that strains of *L. sphaericus* that express the flagellar antigens H 5a5b, H 6 or H 25 are highly toxic to *Cx. pipiens* (Table 46.12). The academic value of such information is less because the extent of expression of the toxin genes is unknown.

The difference between *Cx. quinquefasciatus* and *St. aegypti* larvae in sensitivity to *L. sphaericus* was not due to differences in rates of its ingestion, solubilization, or activation by midgut proteases (Aly *et al.*, 1989). The affinity of cell-surface receptors for binary toxin may be a key factor in determining its toxicity because, as noted above, binary toxin appears not to bind to the midgut epithelial cells or brush border membrane of *St. aegypti* (Davidson, 1989; Nielsen-LeRoux and Charles, 1992).

Scrutiny of the imprecise estimates of the mosquito larvicidal activity of different strains of *L. sphaericus* given in Table 46.9 suggests that Bin is responsible for most of the 1000-fold greater toxicity of strains that have high larvicidal activity compared with those with low larvicidal activity. An attempt to determine the relative toxicities of Mtx1 and Bin to *Cx. quinquefasciatus* larvae was made by bioassays with cultured cells from both a recombinant strain of *E. coli* producing Mtx1 and a recombinant strain of *B. t. israelensis* containing the *bin* gene from *L. sphaericus*. The LD_{50} values of the Mtx1 and Bin sources after 48 h exposure of third- and fourth-instar larvae were not dissimilar (Table 46.13A). However, the delayed effects from 48 h exposure to Mtx1 were much greater than the comparable exposure to Bin (Table 46.13B) (Wei *et al.*, 2006). Unfortunately, the relative amounts of Mtx1 and Bin produced by strains of *L. sphaericus* that produce both are not known.

(b) Resistance

Certain strains of *L. sphaericus* that are highly pathogenic to *Cx. pipiens* and *Cx. quinquefasciatus* have been used as microbial insecticides to control wild populations of those species (de Barjac and

Table 46.13 Acute toxicity and delayed effects from exposure of *Culex quinquefasciatus* larvae to cultured cells from a recombinant strain of *Escherichia coli* containing *mtx1* or from a recombinant strain of *Bacillus thuringiensis israelensis* containing *bin* from *Lysinibacillus sphaericus*. (From Wei *et al.*, 2006.)

A. Acute toxicity against fourth-instar larvae exposed for 48 h

Toxin	LD_{50} (mg l^{-1})*	LD_{90} (mg l^{-1})*
Mtx1	0.65	2.00
Bin	1.70	2.78

B. Delayed effects induced in survivors from 48 h exposure of second- and third-instar larvae to the acute LD_{50}

Toxin	*Pupal death rate (%)*	*Abnormal at emergence (%)*
Control	4.4	1.2
Mtx1	70	20
Bin	6.0	5.0

*, mg dry weight of cultured cells l^{-1}.

Sutherland, 1990), and this has led to the appearance of resistance to *L. sphaericus* in mosquito populations in different geographical regions. More than one resistance mechanism has been described.

A colony of *Cx. quinquefasciatus* from a Californian laboratory, when subjected to selection with LC_{94}–LC_{98} dosages of *L. sphaericus* (strain 2362), developed more than 10^5-fold resistance to binary toxin. Resistant larvae lacked a functional receptor for the toxin, and specific binding could not be demonstrated. Brush border membrane fractions from the F_1 larvae of a cross between susceptible and resistant lines of *Cx. quinquefasciatus* showed binding characteristics similar to those of the susceptible parent, consistent with the resistance being recessive (Nielsen-LeRoux *et al.*, 1995).

Three resistance factors have been found in the western Mediterranean region. Two, from Port-St-Louis and Perpignan in southern France, were named $sp\text{-}1^R$ and $sp\text{-}2^R$, respectively; the third, from Tunisia, was named $sp\text{-}T^R$. All three mutant genes were recessive and sex linked. Larvae homozygous for any one of the three mutations showed >5000-fold resistance. In larvae that were double heterozygotes ($sp\text{-}1^{RS}sp\text{-}2^{RS}$) of the two French mutants, the resistance was >100-fold. Experiments with [^{125}I]-labelled binary toxin and brush border membrane fractions showed that resistance endowed by $sp\text{-}1^R$ was not associated with any loss of affinity for receptors on microvilli. In a comparison of binding kinetics among nine *Culex* strains from different geographical locations, the highest affinity for binary toxin was displayed by brush border membrane fractions from homozygous $sp\text{-}1^{RR}$ and $sp\text{-}T^{RR}$ larvae. An absence of specific binding was recorded in $sp\text{-}2^R$ larvae and in larvae of a Californian strain. With 50 nM [^{125}I]-labelled binary toxin, the specific binding of $sp\text{-}2^R$ was only 6% of that with $sp\text{-}1^R$ and $sp\text{-}T^R$. No saturation could ever be established, and non-specific binding exceeded specific binding whatever the concentration of binary toxin (Nielsen-LeRoux *et al.*, 1997, 2002; Chevillon *et al.*, 2001).

Resistance developed in lines of *Cx. quinquefasciatus* selected with *L. sphaericus* strains C3-41 or 2362; its inheritance was recessive, autosomal and monofactorial. In both colonies, resistance resulted from the failure of binary toxin to bind to its specific receptor (Oliveira *et al.*, 2004).

46.6.7 Pathogenesis in mosquito larvae

Pathogenesis in mosquito larvae infected with *L. sphaericus* varies with the strain of the bacterium and the types and quantities of toxin that it produces. We first consider the pathogenic effects of two strains of *L. sphaericus* that produce mosquitocidal toxins, but not binary toxin (Table 46.9).

The first strain of *L. sphaericus* to have been isolated that affected mosquito larvae was obtained from moribund *Culiseta incidens* larvae found in freshwater rock holes in Fresno County, California. This strain, later named Kellen, was only weakly larvicidal. After 3 days of exposure to the bacterium, fourth-instar larvae of *Cs. incidens* showed loss of turgor and of motility. At that time,

the alimentary canals of the larvae were filled with vegetative cells of *L. sphaericus*, and the cells of the posterior stomach underwent a progressive degeneration. Later, disruption of epithelial cells caused perforation of the midgut, and bacteria entered the haemocoel. The larvae became dark brown and after about 7 days of exposure died, often remaining attached to the surface membrane. Highly motile, vegetative bacilli were distributed throughout the bodies of dead larvae, and sporulating stages were found in dead pupae. Kellen *et al.* (1965) considered this strain to be a facultative pathogen of mosquito larvae.

The strain SSII-1 exhibits moderate larvicidal activity. Within 30 min of ingestion by second- or third-instar larvae of *Cx. quinquefasciatus*, cells of *L. sphaericus* within the anterior and central midgut lumen were lysed, releasing their contents, and this was taken to be the mode of release of a bacterial toxin. Larvae that had been exposed to *L. sphaericus* for a period of 15 min became sluggish 10–12 h later. At 10 h after feeding, vacuoles appeared in cells of the midgut epithelium, and the midgut swelled progressively until it pressed against the outer body wall, eliminating most of the haemocoelic space. By 12 h, cells of the posterior stomach had started to lyse and slough. All larvae were moribund or dead by 24 h, when the cellular structure of the midgut epithelium was markedly disrupted but the basal lamina remained intact. All bacteria were confined within the peritrophic matrix until after the death of the host (Davidson, 1979).

L. sphaericus (strain 2297) has both *mtx* and *bin* genes. In an investigation of the effects of that strain on different species of mosquito, fourth-instar larvae of *Cx. pipiens*, *An. stephensi* and *St. aegypti* were placed in spore/crystal suspensions of appropriate concentration (5×10^{-5}, 5×10^{-3} and 5×10^{-1} mg powder ml^{-1}, respectively). Within 15–35 min of ingestion by all three species, the crystal proteins were solubilized within the anterior stomach. During the first 24 h, *L. sphaericus* spores germinated within the midgut lumen, and after 36 h most bacteria were in the vegetative stage. With *Cx. pipiens* larvae, ultrastructural changes were first observed 35 min after ingestion of the bacteria. Very large vacuoles appeared within the epithelial cells – sequentially in the gastric caeca, anterior stomach and posterior stomach – but only a few cells had lysed when the larvae began to die. With *An. stephensi* larvae, regions of low electron density appeared within the cells of the gastric caeca and posterior stomach 15 min after feeding, and the cell ultrastructure was further modified during the next 24 h. When the larvae began to die, 36 to 48 h after first exposure, almost all midgut cells exhibited swollen mitochondria but only a few cells had lysed. With *St. aegypti* larvae, 1 h after exposure to a high dosage of the spore/crystal complex, ultrastructural changes appeared in the mitochondria and smooth endoplasmic reticulum of the midgut cells, but no other changes occurred within those cells until larval death (Charles, 1987).

The cause of death of mosquito larvae intoxicated only by the Bin protein of *L. sphaericus* remains undecided. Unlike the mosquitocidal toxins of *L. sphaericus* and the *δ*-endotoxins of *B. thuringiensis*, binary toxin does not cause cell lysis in target cells in the mosquito larval midgut, and the midgut epithelial cells of infected larvae appear more or less intact. It is noteworthy that the strains of *L. sphaericus* that possess high mosquito larvicidal activity produce both mosquitocidal toxins and binary toxin (Table 46.9).

Most investigators examined mosquito larvae for up to 48 h after first exposure to *L. sphaericus* toxins. When the period of surveillance was longer, the delayed effects of toxin action became apparent, e.g. increased death rates during the pupal stage and abnormalities in emerging adults (Table 46.13B). Individuals that survived exposure to Mtx1 or Bin only to succumb days later did not carry a live remnant of the *L. sphaericus* infection, so the delayed effects possibly reflected a failure to repair the damaged midgut (Wei *et al.*, 2006).

46.6.8 Field experiments

(a) Persistence

Lysinibacillus sphaericus is widely distributed in soil and in aquatic habitats, and is thought to be

predominantly saprobic. However, with the metabolic cost of toxin production, pathogenesis must play a significant role in its perpetuation. No observations have been made on the dynamics of natural infestations of mosquito populations with *L. sphaericus*, but field experiments have provided information on the multiplication of *L. sphaericus* in nature and on how it may survive from one season to the next. The spore and parasporal body of each cell remain together within the exosporium after cell lysis (unlike those of *B. thuringiensis*). Spore/crystal powders added to water do not sink immediately, but soon settle out. In one experiment, a powder preparation of strain 1593 was introduced at low density into a street catch basin (or storm drain sump) with standing water. Bioassay of water samples revealed negligible toxicity in the surface water after 7 days, but moderately high toxicity persisting at the bottom of the catch basin for 21 days. Natural reinfestation of the catch basin by *Cx. quinquefasciatus* soon occurred, apparently because the larvae did not feed from the bottom. A bacterial suspension in water that was exposed to sunlight did not survive 6 h of exposure (Mulligan *et al.*, 1980).

When dry, spore/crystal preparations of *L. sphaericus* were applied to the surfaces of artificial pools. Most of the particles fell through the water and settled on the muddy substratum within 4 days. Relatively large numbers of viable spores were present in the bottom mud for at least 21 days. However, treated pools that were allowed to dry and then re-flooded developed large populations of mosquito larvae (Davidson *et al.*, 1984). That the spores of *L. sphaericus* can percolate deep into mud and survive there was demonstrated by the removal of mud cores, 15–17 cm long, from a ditch that had been treated 9 months earlier with an *L. sphaericus* culture. Although the ditch had repeatedly flooded and dried out, samples from the top and bottom of the cores contained *L. sphaericus*. Whether mosquito larvae could survive in the re-flooded ditch was not tested (Hertlein *et al.*, 1979). Possibly, soil that harbours spores must be stirred into overlying water to make the spores available for ingestion by mosquito larvae. Stirring the bottoms of catch basins that had been treated with *L. sphaericus* several weeks earlier rendered water toxic that otherwise was non-toxic (Mulligan *et al.*, 1980).

To measure the persistence of *L. sphaericus* (strain 1593) in the presence of mosquito larvae, a container was seeded once with a spore/crystal preparation and repeatedly with *Cx. quinquefasciatus* larvae. The container (volume 20 l, depth 20 cm) was covered with a thin sheet of cloth and kept outdoors in a shaded position. Every 7 days all larvae were removed, whether alive or dead, and replaced. Throughout a 6-month period *L. sphaericus* maintained a relatively high density in the container, but by the end of each 7-day period the *Culex* larvae had suffered 90% mortality. It appeared that the bacteria multiplied in the larval cadavers (Silapanuntakul *et al.*, 1983). In field experiments with *L. sphaericus* strains 1593 and 2362, the bacteria multiplied greatly within cadavers of *Cx. tarsalis*, and 3 days after infection each larva contained 10^5–10^6 spore-containing *L. sphaericus* cells. Davidson *et al.* (1984) concluded that that number of cells would be produced within each infected larva, regardless of the dose ingested, and that multiplication within cadavers may permit repeated cycles of bacterial development in the field when large numbers of susceptible larvae are present. How *L. sphaericus* appears in sufficient density to infect mosquito larvae at the start of a new season is unknown.

(b) Toxicity to mosquito larvae and other aquatic organisms

A 3-year study was undertaken in south-eastern Wisconsin to assess the effects of a commercial formulation of *Lysinibacillus sphaericus* (Vectolex® CG) on mosquito larvae and a wide range of other aquatic invertebrates. Aquatic habitats of two vegetation types were used: canary grass (*Phalaris arundinacea*) marsh and cat's tail (*Typha* sp.) marsh. The canary grass marsh yielded 110 invertebrate taxa and the cat's tail marsh 115. In total, 138 invertebrate taxa were exposed to the insecticide. The microbial insecticide was applied from a helicopter at the recommended dosage.

In each area, a number of samples containing vegetation, detritus, sediment from the substratum and associated invertebrates were collected with D-frame aquatic nets 1 day before and 72 h after spraying. The samples were scored for five characteristics: (i) mean taxa richness (mean number of all taxa); mean diversity (taxa richness and abundance); (iii) dipteran richness (minus mosquitoes) as a proportion of all other taxa richness; (iv) dipteran abundance (minus mosquitoes) as a proportion of all other invertebrate abundance; and (v) changes (as percentages) in feeding classes – collector gatherers, collector filterers, scrapers, shredders and predators.

Weekly sampling for mosquito larvae coupled with a light-trap survey throughout the 3-year study showed a 90–99% reduction in target mosquito species resulting from the microbial insecticide. For the invertebrates other than mosquito larvae sampled, no significant changes were measured in any of the five characteristics detailed above. There were no significant differences in feeding-class composition due to the bioinsecticide in either marsh habitat (Merritt *et al.*, 2005). The authors commented that their finding that *L. sphaericus* formulated as an insecticide produced no detrimental effects on non-target organisms was consistent with the results of earlier investigators.

46.7 *CLOSTRIDIUM BIFERMENTANS*

46.7.1 Systematics and biology

The genus *Clostridium* Prazmowski 1889, family Clostridiaceae and 'phylum' Firmicutes, is a heterogeneous group of approximately 100 known species. They are anaerobic, spore-forming, rod-shaped, Gram-positive and usually motile bacteria, which occur in soil and water and, in some cases, in the alimentary canal of vertebrates. *Clostridium* and *Bacillus* share many phenetic characteristics; but, whereas the species of *Clostridium* are obligatory anaerobes, those of *Bacillus* are aerobic or facultatively anaerobic. Certain species of *Clostridium* are pathogens of higher animals. Botulinus toxin, a mixture of exotoxins, is produced by *Clostridium botulinum*.

Two serovars of *Clostridium bifermentans* isolated from soil samples collected in Malaysia were found to be pathogenic to mosquito larvae. Each had a specific H antigen. One serovar obtained from mangrove swamps was named *C. bifermentans* serovar *malaysia* (de Barjac *et al.*, 1990; Lee and Seleena, 1990). Often, its name is abbreviated to *Cbm*. The other serovar was obtained from secondary forest and was named *C. bifermentans* serovar *paraiba* (Seleena *et al.*, 1997).

Cells of *C. bifermentans* serovar *malaysia* produce exotoxins and endotoxins at different times during the growth cycle: the exotoxins during the exponential phase and the endotoxins during the stationary phase when sporulation occurs. Under the culture conditions used by Seleena and Lee (1995), at 37°C, the lag phase lasted for about 3 h post-inoculation, and the exponential phase lasted from approximately 4 h until 8 h post-inoculation (Figure 46.17). Under the different conditions used by Charles *et al.* (1990), and at 34°C, the generation time during exponential growth was *c.* 30 min, and the period of exponential growth lasted 2–3 h. At the time that the exponential phase ended, initiation of a forespore septum was apparent in 5% of cells. Sporulation was achieved, with sporangium lysis, within 6–9 h after the end of the exponential phase. A number of feather-like structures, totalling 12 at most, extended from each end of the spore. During sporulation, parasporal bodies appeared within the cell cytoplasm. Unlike the equivalent structures in *Bacillus thuringiensis*, these did not have a crystalline ultrastructure and were not surrounded by an envelope.

46.7.2 Putative exotoxins

Three investigations provided evidence of exotoxin secretion. (i) The cells of *C. bifermentans* serovar *malaysia* (*Cbm*) were non-toxic to cockroaches (*Blatella germanica*), but daily ingestion of sterile, filtered supernatant led to a 90% death rate over 10 days (Seleena and Lee, 1995). (ii) Exposure of snails (*Biomphalaria glabrata*) to culture supernatant diluted to 1% caused 60–93% mortality. During its growth cycle, *Cbm* secreted a number of organic acids into the supernatant (acetic, 50 mM; pro-

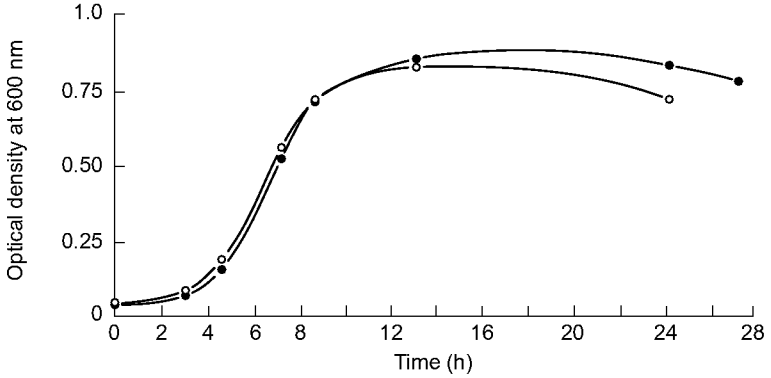


Figure 46.17 Growth curves of two batches of *Clostridium bifermentans* serovar *malaysia* in flask culture. (After Seleena and Lee, 1995.) Inocula of *C. bifermentans* were added to brain-heart infusion broth and incubated at 37°C for 24 or 28 h. Relative cell mass was determined from optical density. The lag, exponential and stationary phases of the growth cycle are shown.

pionic, 5 mM; phenylpropionic, 10 mM; isovaleric, 5 mM; and isocaproic, 20 mM). When diluted to 1%, the acids collectively caused 87.5% mortality among the snails; when tested singly they caused 20–75% mortality (Thiéry *et al.*, 1992). (iii) Many strains of *C. bifermentans* synthesize and excrete highly active proteinases, mainly during vegetative growth. In batch cultures of *Cbm*, extracellular proteolytic activity was due mainly to metallo-proteinases and/or cysteine proteinases, which were highly active in the pH range encountered in the culture (pH 6.0–7.4) (Nicolas *et al.*, 1990).

46.7.3 Endotoxins

Most studies of toxicity have concerned endotoxins in the parasporal bodies, which, limited tests suggest, are toxic specifically to larvae of nematocerous Diptera. Among the Culicidae, toxicity has been demonstrated to species of *Anopheles*, *Culex* and *Ochlerotatus*. Among other nematocerous families, toxicity has been found to single species of Chaoboridae, Psychodidae and Simuliidae. The preparations were non-toxic to brachyceran Diptera of the genera *Musca* and *Drosophila*, and among other insect orders they were non-toxic to single species of Hemiptera, Ephemeroptera, Coleoptera and Lepidoptera. No toxicity was found to two species of Crustacea and to one of Mollusca (Thiéry *et al.*, 1992; Yiallouros *et al.*, 1994).

In batch cultures, toxicity to *An. stephensi* larvae reached a maximum within 5 h of the start of sporulation, and remained constant until 8 h. When sporangium lysis and spore release started between hours 8 and 9, toxicity was lost, even in whole-culture samples (Charles *et al.*, 1990). That this loss of toxicity resulted from exposure to proteinases excreted earlier into the culture medium was shown by the almost total disappearance of larvicidal activity that occurred when particulates, from cells sonicated after 11 h culture, were incubated with cell-free supernatant obtained at the same time (Nicolas *et al.*, 1990).

Both sporulated cells and isolated parasporal bodies of *Cbm* were toxic to mosquito larvae. When third-instar *An. stephensi* larvae were exposed to dilutions of sporulated cell cultures for 48 h, the LC_{50} was 10^{-6} vol/vol (similar to that of equivalent cultures of *B. thuringiensis israelensis* or *L. sphaericus*). Fourth-instar larvae of *Cx. pipiens* and *St. aegypti* larvae were, respectively, 10× and 100× less sensitive. The LC_{50} to second-instar *An. stephensi* larvae of isolated parasporal bodies was 0.3 μg ml^{-1} (de Barjac *et al.*, 1990).

In another series of assays, cells of *Cbm* were harvested when they contained spores and parasporal bodies. Third-instar larvae of *Anopheles* and fourth-instar larvae of *Ochlerotatus*, *Stegomyia* and *Culex* were placed in suspensions of the cells, and LC_{50} values were determined after 48 h exposure. Of the species tested, the most sensitive was *An. gambiae* (LC_{50} = 2 × 10^5 cells ml^{-1}). *Anopheles*

stephensi, *Ochlerotatus caspius* and *Oc. detritus* were one order less sensitive (LC_{50} 1.6–1.7 × 10^4 cells ml^{-1}). For *St. aegypti* the LC_{50} was 5.3–9.4 × 10^4 cells ml^{-1}; for *Cx. quinquefasciatus* and *Cx. pipiens* it was 12.8 and 16.5 × 10^4 cells ml^{-1}, respectively (Thiéry *et al.*, 1992).

Suspensions of lysed and lyophilized bacteria were used to measure and compare the toxicities of the *C. bifermentans* serovars *malaysia* and *paraiba*. Both serovars were active against *Anopheles maculatus* larvae, but showed only low activity against *St. aegypti*; the toxicity to *Cx. quinquefasciatus* was low to absent (Table 46.14) (Seleena *et al.*, 1997).

Genetic analyses of putative insecticidal factors of *C. bifermentans* serovar *malaysia* identified four genes of interest. Two that were situated 96 bp apart, and that initially were named *cbm71* and *cbm72*, were structurally related to the *δ*-endotoxin genes of *Bacillus thuringiensis*. They encoded, respectively, proteins of deduced molecular mass 71.13 and 71.73 kDa, which now are included in the 'List of *Bacillus thuringiensis* Holotype Toxins' under the names Cry16Aa and Cry17Aa (Barloy *et al.*, 1996, 1998; Crickmore *et al.*, 2006). Two short open reading frames found 2771 bp downstream of the *cry17Aa* stop codon were variants of a single gene. One, named *cbm17.1*, encodes a protein with a deduced molecular mass of 17.19 kDa (Cbm17.1). The other, *cbm17.2*, encodes a protein with a deduced molecular mass of 17.45 kDa (Cbm17.2) (Table 46.15). These two proteins share 44.6% similarity with the haemolysin of *Aspergillus fumigatus*, but in preliminary tests did not show haemolytic activity (Barloy *et al.*, 1996, 1998).

The larvicidal activity of Cry16Aa was investigated after *cry16Aa* had been expressed in a non-toxic strain of *B. thuringiensis*. The transformants produced no parasporal inclusion during sporulation. When the Cry16Aa that had been synthesized during sporulation and excreted into the supernatant was precipitated with trichloracetic acid and assayed against second-instar larvae, the LC_{50} values were: *An. stephensi*, 129 μg ml^{-1}; *St. aegypti*, 185 μg ml^{-1}; and *Cx. pipiens*, 156 μg ml^{-1} (Table 46.15). Barloy *et al.* (1996) surmised that these low levels of toxicity might have been due to the transformants producing only small amounts of Cbm71, i.e. about 1% of total proteins. With similar protocols, Cry17Aa showed negligible activity, if any. Cbm17.1 and Cbm17.2 showed no larvicidal activity, and also showed no haemolytic activity with sheep and horse erythrocytes (Barloy *et al.*, 1998). Later, Juárez-Pérez and Delécluse (2001) found no mosquitocidal activity in recombinant Cry16Aa, Cry16Aa, Cbm17.1 or Cbm17.2, and concluded that the mosquitocidal activity of *C. b.* serovar *malaysia* is due to other, unknown factors.

46.8 *SPIROPLASMA*

46.8.1 Characteristics and classification

As classified in *Bergey's Manual* (Garrity, 2005), the genus *Spiroplasma* Saglio 1973 is the sole genus of

Table 46.14 Toxicities of two serovars of *Clostridium bifermentans* to three species of mosquito larvae. (After Seleena *et al.*, 1997.)

		Toxicity (LC_{50})		
Serovar	*Flagellar antigen*	Anopheles maculatus	Culex quinquefasciatus	Stegomyia aegypti
C. b. malaysia	CH 18	2.6	> 3000	400
C. b. paraiba	–	38	110	740

LC_{50} of third or fourth-instar larvae after 24 h exposure, expressed as μg l^{-1} of a suspension of powder produced by lyophilizing lysed bacteria.
The LC_{50} of *Bacillus thuringiensis* serovar *israelensis* (strain IPS82) to *An. maculatus* larvae, when prepared and tested in the same way, was 19 μg l^{-1}.

Table 46.15 Genes of putative toxins of *Clostridium bifermentans* serovar *malaysia* and their encoded proteins, with data on toxicity to *Culex pipiens* of the same toxins produced in transformed *Bacillus thuringiensis*. (From the data of Barloy *et al.*, 1996, 1998.)

Name of gene		*Protein*		Culex pipiens
Provisional	*Formal*	*Name*	*kDa*	LC_{50} ($\mu g\ ml^{-1}$)
cbm71	*cry16Aa*	Cry16Aa	71.13	156 (±10)
cbm72	*cry17Aa*	Cry17Aa	71.73	Non-toxic
cbm17.1	–	Cbm17.1	17.19	Non-toxic
cbm17.2	–	Cbm17.2	17.45	Non-toxic

the family Spiroplasmataceae, which falls within the phylum 'Firmicutes' and class Mollicutes (cf. Section 46.1.1). Through regressive evolution and genome reduction, the Mollicutes became the smallest and simplest free-living, self-replicating forms of life, and share the following major characteristics. (i) Lack of a cell wall; only a cholesterol-containing unit membrane envelopes the cells (for which reason they do not respond to Gram's stain); cholesterol, which occurs uncommonly in bacteria, increases the rigidity of the membrane. (ii) Lack of flagella; spiroplasmas are, however, capable of motility and chemotaxis. (iii) Possession of an internal contractile cytoskeleton, which functions as a linear motor. (iv) Small, deformable cells that pass through membrane filters of ~220 nm pore size. (v) Relatively small genomes with a low G + C content (mostly below 30%) and high A + T content. Bacteria that after triple cloning are insensitive to penicillin and bound only by a cell membrane are placed in the class Mollicutes.

The first spiroplasma to be isolated and described, giving a name to a new genus and its type species, was *Spiroplasma citri*, a mycoplasma-like organism present in the sieve tubes of citrus plants in Morocco and California that were affected by 'Stubborn' disease (Saglio *et al.*, 1973). The *List of Prokaryotic Names with Standing in Nomenclature* (Euzéby, 2011) includes 37 species of *Spiroplasma*. In previous publications, 36 species had been listed and separated into 29 serogroups, based on the cross-reactivities of their surface antigens. Three of the serogroups were further divided into subgroups of from two to six species (Tully *et al.*, 1987; Williamson *et al.*, 1998; Gasparich *et al.*, 2004).

Examination of all relevant data indicated a close similarity between classifications of *Spiroplasma* based on serological or molecular characteristics. A phylogram resulting from cladistic analysis of 16S rRNA sequences showed the positions of clades at higher level hierarchies of the class Mollicutes, one of which was the Spiroplasma–Entomoplasmataceae–Mycoides (SEM) clade. All trees and bootstrap values for the major nodes supported successive division of the SEM clade into four smaller clades. From a common ancestor, the first branching separated a basal ixodetis clade (*Spiroplasma ixodetis* only) from three other clades. At the next node the Citri–Chrysopicola–Mirum clade (of 9 species of *Spiroplasma*) was separated from the remaining species. At the final node two sister clades were separated: (i) the Apis clade (of 26 species of *Spiroplasma*) and (ii) the Mycoides–Entomoplasmataceae clade (of 12 species of *Mycoplasma, Entomoplasma* or *Mesoplasma*). Gasparich *et al.* (2004) concluded that all currently classified *Spiroplasma* species form a single evolutionary unit which is derived from a common ancestor, and that the fact that the unit is paraphyletic should not affect the position of *Spiroplasma* in the construction of Linnean classifications.

Characteristics that allow identification of specimens as *Spiroplasma* are: (i) a basic, helical cell geometry at some stage of the life cycle; (ii) possession of an internal cytoskeleton in the form of a flat fibrillar ribbon attached to the inside of the

cellular tube; and (iii) motility that results from the helical form and actions of the cytoskeleton (Whitcomb *et al.*, 1995; Trachtenberg, 2004, 2006). All known species of *Spiroplasma* are filterable through a 220 nm filter, are resistant to penicillin (500 u ml^{-1}), ferment glucose, and are unable to hydrolyse urea. Most species have a sterol requirement for growth (Regassa and Gasparich, 2006, review).

Mollicutes function with some of the smallest genomes known in self-replicating organisms. The smallest, of 577 to 590 kbp, is that of *Mycoplasma genitalium* (Su and Baseman, 1990), which is less than twice the size of a large viral genome such as that of a poxvirus (Section 43.2.6). At one time, genome size was considered a highly conserved trait among mollicutes, but it has been shown that mollicute genomes vary widely in size and can apparently change over short periods of evolutionary time. Within the genus *Spiroplasma*, genome sizes range from 940 to 2220 kbp (Carle *et al.*, 1995; Gasparich *et al.*, 2004).

46.8.2 Host–spiroplasma relationships

Most known species of *Spiroplasma* live in association with insects – of at least seven orders; a few species are associated with ticks or plants, and isolates have been obtained from crustaceans. In arthropod hosts, spiroplasmas typically attach to the apical surface of cells of the midgut epithelium. Most species multiply within the midgut lumen without any apparent adverse effect on the host, and appear to be commensals. However, a few species invade internal organs and are pathogenic (Section 46.8.6). Certain spiroplasmas that are transmitted transovarially cause sex ratio disorders and kill the male progeny of their hosts. This is best known in the case of *Spiroplasma poulsonii* which infects *Drosophila willistonii*.

A small number of species have been isolated from plants, with which the relationship may be mutualistic or parasitic. It has been surmised that some spiroplasmas occur in nectar and infect certain insects that imbibe the nectar, but that is inconsistent with the almost complete failure to isolate spiroplasmas from male mosquitoes. Dispersal and transmission of spiroplasmas on plant surfaces is not known to adversely affect the plant, but infection of internal plant tissues is pathogenic. Three species have obligate insect/plant transmission cycles and are phytopathogens. One is *Spiroplasma citri* (sub-group I-1), the causal agent of Citrus Stubborn disease. The phytopathogens are maintained in their hemipteran hosts which, during feeding, transmit them in saliva to the plant phloem where further replication occurs (Regassa and Gasparich, 2006).

46.8.3 Spiroplasmas that infect mosquitoes

Spiroplasmas isolated from mosquitoes have been found only in the adults, and essentially only in adult females. Isolates could not be obtained from external washings from mosquitoes, but were obtained from whole-body homogenates. How adult mosquitoes become infected with spiroplasmas is not known. The symbiotic association appears to be obligatory for the spiroplasmas, and therefore is classed as parasitic.

Five species of *Spiroplasma* have been isolated from mosquitoes (Table 46.16). *Spiroplasma culicicola* was first isolated from a pool of adult female *Ochlerotatus sollicitans* collected in New Jersey in August 1981. No spiroplasmas were present in surface washes of flowering, salt-marsh plants collected at the same time (Slaff and Chen, 1982; Hung *et al.*, 1987). *Spiroplasma sabaudiense* was first isolated from a mixed pool of *Aedimorphus vexans* and *Oc. sticticus* collected while feeding on humans in the northern French Alps in 1983 (Abalain-Colloc *et al.*, 1987). Later, *S. sabaudiense* was isolated from *Oc. caspius* and *Ochlerotatus detritus* caught on the banks of the Loire and the Atlantic coast of France (Le Goff *et al.*, 1990). Serological tests on group XVI spiroplasmas isolated from jointly pooled *Am. vexans* and *Ochlerotatus cantans*, collected in the Savoy region of France, yielded a strain (Ar-1357) which represented a new species of *Spiroplasma* but which was not given a species name (Abalain-Colloc *et al.*, 1993).

A new spiroplasma was isolated from a pool of

Table 46.16 Named and an unnamed species of *Spiroplasma* that have been isolated from mosquitoes.

Species	*Original isolates*		*Strain*	*Group*	*Refs* †
	Hosts *	*Location*			
S. culicicola	*Oc. sollicitans*	New Jersey, USA	AES-1^T	X	1
S. sabaudiense	*Am. vexans/Oc. sticticus*	Savoy, France	AR-1343^T	XIII	2
Unnamed	*Am. vexans/Oc. cantans*	Savoy, France	Ar-1357	XVI-3	3
S. taiwanense	*Cx. tritaeniorhynchus*	Taishan, Taiwan	CT-1^T	XXII	4, 5
S. diminutum	*Cx. annulus*	Taishan, Taiwan	CUAS-1^T	XXV	6

*, *Ochlerotatus sollicitans, Aedimorphus vexans, Ochlerotatus sticticus, Ochlerotatus cantans, Culex tritaeniorhynchus, Culex annulus*.
†, References to the original isolations or descriptions of the species; 1, Hung *et al.* (1987); 2, Abalain-Colloc *et al.* (1987); 3, Abalain-Colloc *et al.* (1993); 4, Clark *et al.* (1987); 5, Abalain-Colloc *et al.* (1988); 6, Williamson *et al.* (1996).
T, Type strain of the species.
/, Indicates mixed pool of two host species.

100 female *Culex tritaeniorhynchus* that had been attracted to animal bait on 2 July 1981 at Taishan in Taiwan, and this was named *Spiroplasma taiwanense* (Clark *et al.*, 1987; Abalain-Colloc *et al.*, 1988). Spiroplasmas present in a triturate of 83 female *Culex annulus* collected at Taishan on 5 September 1980 were designated strain CUAS-1 and the triturate was frozen. Much later, the triturate was processed, the spiroplasmas were characterized, and the strain was given species status with the name *Spiroplasma diminutum* (Williamson *et al.*, 1996). At the same time, another pool was analysed of female *Cx. tritaeniorhynchus* that had been collected in Taishan on 5 September 1980, the same day as *Cx. annulus* (above), and this yielded isolates of *S. diminutum* (Williamson *et al.*, 1996). A number of other isolates of *Spiroplasma* from mosquitoes have been insufficiently characterized to be given strain or species status. One example, characterized by its 16S rRNA sequences, was isolated from *An. funestus* in Kenya (Lindh *et al.*, 2005).

In liquid cultures, the spiroplasmas isolated from mosquitoes assumed a helical form, but only rarely in the case of *S. culicicola*. The helical cells of *S. culicicola* and *S. diminutum* are 1–2 μm long, but, whereas the cells of *S. culicicola* can be seen to have about one to two turns, those of *S. diminutum* appear to be spheroidal, rapidly moving bodies. The cells of *S. sabaudiense* and *S. taiwanense* are 3.1–3.8 μm long and have more than two turns. All four of these *Spiroplasma* species are ~100–200 nm in diameter. They are pleiomorphic, and all pass through membrane filters with pore diameters of 220 nm, but not through those with pores of 100 nm. The optimal temperature for growth of three of the four species is 30°C to 31°C; only *S. diminutum* can grow at 37°C (Abalain-Colloc *et al.*, 1987, 1988; Hung *et al.*, 1987; Williamson *et al.*, 1996).

46.8.4 Ultrastructure

A spiroplasma cell is not bounded by a cell wall but by a relatively rigid unit membrane. It is tubular in form but helically coiled. The cytoskeleton of *Spiroplasma melliferum* was shown by negative staining to be a flat, contractile ribbon composed of seven fibrils attached to the inner side of the cell membrane along its shortest helical line. Both the tubular cell and the cytoskeletal ribbon are coiled into a dynamic helix, the ribbon following the shortest (inner) helical line on the inner surface of the cellular tube. On average, the cells have five helical repeats, each with a length of c. 0.9 μm (Figure 46.18).

The seven fibrils are the functional units of the contractile cytoskeletal ribbon. Each fibril consists of two aligned filaments, with a combined width of ~9–10 nm. The filaments are composed of

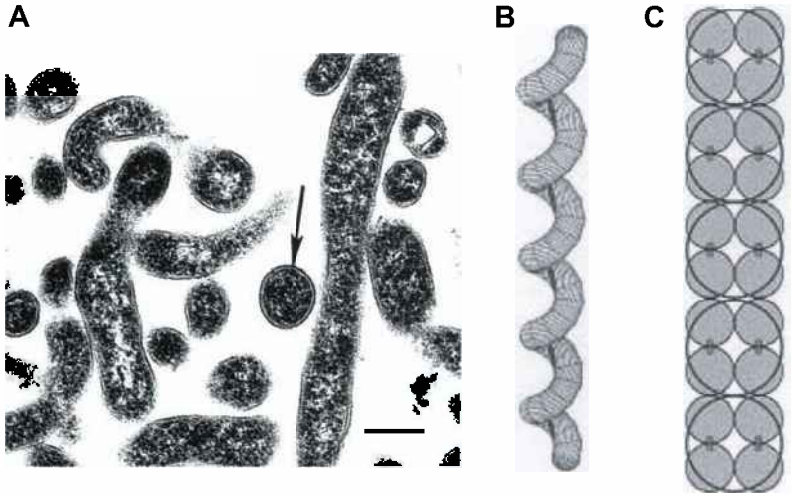


Figure 46.18 *Spiroplasma*: structure and models. **A.** Cells of *Spiroplasma* sp., strain BARC 1901, group XXXIV, from a horsefly (Diptera, Tabanidae). (From Williamson *et al.*, 1998.) Electron micrograph of cells in the exponential growth phase, centrifuged from the culture medium, fixed and sectioned. The arrow indicates a unit cell membrane. Scale bar, 200 nm. **B.** Model of a *Spiroplasma* cell with average helical parameters and at a nearly stretched state. (After Trachtenberg, 2004.) The interrupted vertical line represents the flat cytoskeletal ribbon, which follows the shortest helical line inside the coiled, cylindrical outer membrane. **C.** Model of a functional fibril, which consists of two filaments composed of fib monomers of ~5 nm diameter (grey circles). The pairs of monomers are of opposite polarities, and neighbouring pairs in each filament form tetrameric rings of ~10 nm diameter (large circles).

monomers of 59 kDa, expressed by the *fib* gene. The monomers are arranged in pairs with opposite polarities, and neighbouring pairs form a tetrameric ring of ~10 nm diameter (Figure 46.18C). A fully expanded tetramer is of ~10 nm diameter, allowing for 100 tetramers/μm. Motive force is thought to be generated by a conformational change of the tetrameric subunit from a circular to an elliptical arrangement. The axial length of the rings may shorten by ~40%, driving the changes in fibril lengths and consequently the helical dynamics. *Spiroplasma* cells are dynamic helices; the state of strict helical symmetry exists only briefly, and from it other asymmetric helical forms are derived (Trachtenberg, 2004, 2006).

46.8.5 Locomotion

A fundamental characteristic of bacterial propulsion, due to their small size, is the very low Reynolds number (typically ~10^{-5}). The Reynolds number is a dimensionless number that expresses the ratio of the magnitudes of the inertial force and the viscous force (Volume 1, Section 4.3.1). For microorganisms, inertial effects are small, momentum has no role, and movements are dominated by viscous forces. Most bacteria move by gliding over a substratum or swimming by using forces generated by one or more flagella driven by rotary motors, mechanisms that function readily at a low Reynolds number. However, to move and to change direction, the helical *Spiroplasma* cells need to deviate repeatedly from plain helicity, using mechanisms that break the symmetry.

The *Spiroplasma* cell's geometry and dynamic helical parameters, and so its motility, can be controlled by changing the length of the fibrils differentially and in a co-coordinated manner. In that way the fibrils function as linear motors. Consider a straight tube with two parallel fibrils along one side. If both fibrils shorten the tube curves towards the side with fibrils, and if both

lengthen the tube curves away from that side. If only one fibril shortens, the tube bends symmetrically away from the unshortened fibril. The position of the ribbon along the shortest helical line in the cellular tube maximizes the efficiency of the linear motor, because small changes in length in this location have large effects on cell shape. Propagating local rather than uniform length changes generates non-helical dynamics. Local changes in length result in the breaking of helical symmetry and in non-reciprocating cell movements, allowing for net directional displacement. Flexing, i.e. sharp, random bending about an arbitrary point along the cell, causes the cell to change its swimming direction (Gilad *et al.*, 2003; Trachtenberg, 2004).

Digital-image data of swimming *S. melliferum* confirmed those principles and revealed that helical deformations travel along the cell at up to ~40 μm s^{-1}. In a medium of 1.147 centipoise viscosity the cells had a Reynolds number of ~3.5×10^{-6}, 'ran' at ~1.5 μm s^{-1}, and consumed ~30 ATP molecules s^{-1} (Gilad *et al.*, 2003).

Shaevitz *et al.* (2005) studied the motion of free-swimming *S. melliferum* cells by differential interference contrast microscopy with computer analysis, and his findings appear consistent with the model. Swimming was driven by large 'kinks' propagated along the tubular cell from anterior to posterior. Propagation of a kink resulted from a change in helicity of the cell body moving from anterior to posterior. As one kink approached the posterior end of the cell, a new kink appeared anteriorly. The result was the movement of waves posteriorly along the cell. Fluid within the boundary layer near the cell surface moved posteriorly also, propelling the cell forward. As a kink progressed posteriorly, viscous drag on the longest kink-free portion of the cell caused the cell to change direction.

Observations on the geometry of swimming *Spiroplasma* showed that propulsion results from the propagation of pairs of kinks along the body axis of the cell, the bacterium being propelled by hydrodynamic force as the fluid associated with the kinks moves rearward with the 'body wave'. All kinks start at the same end of the cell, treated as the 'front', and travel towards the other end (the back). As the kink propagates, the cell changes direction through an angle related to the pitch angle of the helix. As a result of the unbalanced viscous drag between different portions of the cell separated by kinks, the cell swims in a zigzag path, rotating about its body axis during kink propagation. Maximum propulsive efficiency was achieved when the pitch angle was *c.* 35.5° and the ratio between inter-kink distance and cell length was *c.* 0.338 (Yang *et al.*, 2009).

46.8.6 Pathogenesis

Some insect-infecting species of *Spiroplasma* are able to move from the initial site of attachment on the midgut epithelium into the haemolymph and thence to other internal organs where they multiply; they are pathogens. Most studies of the pathogenesis of *Spiroplasma* in mosquitoes have involved the unnatural procedure of intrathoracic inoculation into adults. Female *St. aegypti* suffered an increase in death rate over controls from 14 or more days onwards after inoculation with 3.4×10^5 CCU (colour changing units) of *S. culicicola* or 5.1×10^4 CCU of *S. taiwanense* (Vazeille-Falcoz *et al.*, 1994). The flight capabilities of *St. aegypti* and *An. stephensi* were significantly impaired from 3–4 days onwards after inoculation with *S. taiwanense* ($p < 0.01$), and their survival was significantly reduced ($p < 0.001$) (Humphery-Smith *et al.*, 1991b). Five days after adult *An. stephensi* had been inoculated with *S. taiwanensis*, spiroplasmas were present in abundance in the haemolymph, within and between the myofibrils of the thoracic flight muscles, and within the neural lamella and glial cells that surround the nervous system. They occurred at low density in connective tissue, and in cells of the midgut, fat body and tracheae. Replication occurred within the host cells. Later, spiroplasmas invaded the salivary glands and the ovaries. Lysis of host cells permitted the release of spiroplasmas. Three forms of spiroplasma were observed: helices, seen during the exponential growth phase; rounded forms present after exponential growth; and intermediate, pleiomorphic forms (Phillips and Humphery-Smith, 1995).

One comparison has been made of the extent of toxicity resulting from infection by the oral and intrathoracic routes. Females of *St. albopicta* were infected with *S. diminutum* by either intrathoracic inoculation or ingestion in an artificial blood meal. The spiroplasmas replicated strongly and at similar rates after infection of the mosquitoes with small dosages by either route. However, whereas intrathoracic inoculation of 1.7×10^3 infective units per female caused mortality to rise from 14% at day 8 to 97% at day 16, ingestion of 6.6×10^5 infective units had caused no significant mortality over that of the controls by day 16 (Vorms-le Morvan *et al.*, 1991).

Spiroplasmas are not known to infect mosquito larvae in nature, but the addition of *S. taiwanense* to the rearing water of *St. aegypti* larvae significantly reduced the number that reached the pupal or adult stage (Simitzis-Le Flohic *et al.*, 1988; Humphery-Smith *et al.*, 1991a). When cultured in the presence of *St. albopicta* cells, *S. sabaudiense* caused vacuolization and formation of syncytia, and a reduction in growth rate. The degree of cytotoxicity increased with number of passages: whereas only cell lysis was apparent during the third passage, within 6 days after the start of the fourth passage the cell cultures had been destroyed (Humphery-Smith *et al.*, 1988).

All four of the named mosquito-infecting spiroplasmas were non-pathogenic when inoculated intracerebrally or intraperitoneally into suckling mice or rats. *Spiroplasma culicicola* persisted for 14 days in the brain of intracerebrally inoculated mice, but did not replicate (Vazeille-Falcoz *et al.*, 1994).

46.9 *WOLBACHIA*

46.9.1 Introduction

(a) Discovery

During an investigation of rickettsia-like organisms in insects collected in the north-eastern USA, specimens of *Culex pipiens* were among those found to be infected. All of 25 adult male and female *Cx. pipiens* caught in Boston or Minneapolis contained rickettsia-like organisms in their testes or ovaries; none were seen in other organs. The same organisms were present in the eggs and, scantily, in the larvae, but they were present in large numbers in the pupal gonads. This indicated transovarian transmission from one host generation to the next (Hertig and Wolbach, 1924). Later, a new genus, *Wolbachia*, was created for this symbiont, and the species was named *Wolbachia pipientis* (Hertig, 1936). Apart from a brief report of *W. pipientis* infecting *Cx. pipiens* in France (Callot, 1950), it received little or no further attention for over 30 years.

Shortly after *W. pipientis* was named, Marshall (1938) reported that in attempts to make crosses between four autogenous populations of *Cx. pipiens*, three from England and one from France, only one cross had been compatible in both directions. The other crosses were unidirectionally infertile, with the eggs failing to hatch except where the males of a given population mated with the females of certain other populations. Ghelelovitch (1952) commented that, from any cross mating, all F_1 females share the same chromosomal constitution, derived from both parents, therefore the viability of embryos is governed by the cytoplasm, which is derived from the female parent. This was confirmed by backcrosses through nine generations. During the 1950s, this phenomenon was shown to occur with geographically separated populations of *Cx. pipiens* and of *Cx. quinquefasciatus* throughout the world. Some crosses were fertile in both directions, others were infertile in one direction only, and yet others were infertile in both directions. 'Crossing type' was independent of the nuclear genome, its inheritance being controlled by a factor in the cytoplasm which was inherited down the female line. The term 'cytoplasmic inheritance' was adopted for this phenomenon, already known from plants, and later the term 'cytoplasmic incompatibility' was used to indicate sterile crosses (Laven, 1957a,b, 1959, 1967).

In the early 1970s, the presence of *W. pipientis* in the germ cells of *Cx. pipiens* was confirmed by electron microscopy, while crosses between wild-type and tetracycline-treated populations (which had been rendered aposymbiotic, i.e. lacking symbionts) showed *Wolbachia* to be the inherited cytoplasmic factor (Yen and Barr, 1971, 1973). Later, *Wolbachia* was reported from many species of insects, and from arachnids, crustaceans and filarial nematodes.

(b) Symbiosis

A broad definition of symbiosis is 'the living together of two dissimilar organisms in close association'. But two forms of symbiotic lifestyle are observed: if the association benefits one partner only, it is parasitic; if both partners benefit, it is mutualistic. Symbionts and their hosts may experience transitions between the two lifestyles, and can use similar genetic mechanisms in mediating the two forms. The relationships between *Wolbachia* and its hosts are undoubtedly symbiotic.

On an evolutionary time scale, the situation is

thought to be different for rickettsias (including *Wolbachia*) from that for bacteria generally. Rickettsias, being obligate, intracellular (endosymbiotic) bacteria, replicate only within cells of their eukaryote hosts, and have diminished genomes. They are thought to have evolved from a clade of Gram$^-$, aerobic, intracellular α-proto-bacteria that separated from a common ancestor c. 400 million years ago, and that diverged into two lineages: (i) a lineage that now infects only filarial nematodes, living in mutualist associations; and (ii) a lineage that now infects arthropods of three classes and, to a very minor extent filarial nematodes, in usually parasitic associations (Bordenstein *et al.*, 2009).

Studies of interactions between strains of *Wolbachia* and their invertebrate hosts revealed a variety of symbiotic associations – both parasitic and mutualistic, but mostly parasitic. In mosquitoes, the association might be considered parasitic; cytoplasmic incompatibility produces a fitness advantage of *Wolbachia*-infected over uninfected female hosts. With some hosts, *Wolbachia* changes the sex ratio in favour of females. For example, it induces parthenogenesis in some parasitic wasps, causing the females to produce only female offspring from unfertilized eggs. With its isopod hosts, genetic males are converted into reproductively competent females. In contrast, the association of *Wolbachia* with filarial nematodes appears to be beneficial to the hosts (as well as to *Wolbachia*).

46.9.2 Systematics

Many isolates of *Wolbachia* have distinctive nucleotide sequences and are treated as strains. According to their affinities, these strains have been sorted into 'groups' and 'supergroups' within a phylogenetic assemblage.

(a) The genus Wolbachia

Bacteria of the order Rickettsiales are obligate, intracellular occupants of eukaryotic cells. Most rickettsias infect both a vertebrate and a haematophagous arthropod, and rely on their arthropod host and vector for horizontal transmission to their vertebrate host. In contrast, *Wolbachia* is limited to invertebrate hosts (some of which are parasites of vertebrates), and these symbionts are transmitted from one generation to the next of their invertebrate host by transovarian transmission.

A revised classification of Alphabacteria redefined the order Rickettsiales, and placed its species in two families: (i) the Rickettsiaceae (*Rickettsia*, *Orientia*), which inhabit the cytoplasmic compartment of host cells; and (ii) the Anaplasmataceae (*Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*), which inhabit an intravacuolar compartment within the cytoplasm (Dumler *et al.*, 2001). A rooted phylogenetic tree based on 16S rRNA gene sequences shows the four genera of the Anaplasmataceae to form a clade. *Wolbachia* differs from the other genera of Anaplasmataceae by significant differences in its16S rRNA and *groESL* gene sequences, by its absence from vertebrates as hosts, in its association with a wide range of arthropods and with filarial nematodes, and in its highly efficient transovarian transmission (O'Neill *et al.*, 1992; Taillardat-Bisch *et al.*, 2003). The position of the Anaplasmataceae within a phylogenetic classification of the Kingdom Bacteria is described in Section 46.1.1.

(b) Species of Wolbachia

After designation of the genus *Wolbachia* and of *W. pipientis* as type species by Hertig (1936), three other species were assigned to *Wolbachia* (*Wolbachia melophagi*, *Wolbachia persica* and *Wolbachia trichogrammae*), but these have been transferred to other genera (Dumler *et al.*, 2001). Isolates of *Wolbachia* have been grouped into many strains, which, as already noted, have been sorted by their affinities into 'groups' and 'supergroups' (Subsections 46.9.1.c–e below). The taxonomic difficulties inherent in defining bacterial species (Section 46.1.2) are compounded in the case of *Wolbachia*. No strains have been reared in pure culture owing to their very particular requirements, so methods

such as biochemical characterization and DNA–DNA hybridization have not been feasible. With uncertainty over the formal taxonomic status of the many strains, most investigators have referred to them simply as *Wolbachia*, a practice which is not taxonomically legitimate.

In an attempt to find whether the supergroups of *W. pipientis* warranted species status, the nucleotide sequences were determined for the three protein-coding genes *gltA*, *ftsZ* and *groEL* from strains from all known supergroups, and the phylogeny was inferred from the concatenated data set that was rooted with two outgroup species, *Anaplasma marginale* and *Ehrlichia ruminantium*. Using the statistical test of Shimodaira and Hasegawa (1999), which distinguishes between competing hypotheses, the investigators compared the relative support for 18 topologies in which the root of the tree (leading to the two outgroups) was placed as a sister group to each of the different supergroups. From their likelihood values these phylogenies were statistically indistinguishable, i.e. no one root placement was significantly more likely than any other. The investigators considered that without a reliable root for the tree they could not confidently give any supergroups species rank, and decided that it was more prudent to treat all strains as *W. pipientis* (Casiraghi *et al.*, 2005; Lo *et al.*, 2007).

The facts that among wolbachiae that infect arthropods phage sequences can be transferred horizontally between different strains, and that recombination is widespread (Section 46.9.3.b,c) are consistent with all of these strains being members of one species, *W. pipientis*. The strains of *Wolbachia* that infect filarial nematodes differ in genetic composition and biological characteristics from those that infect arthropods. Where known, the genomes of the nematode-infecting strains are smaller and show little or no recombination. Horizontal transfer of *Wolbachia* between nematode hosts has not been observed in nature or in the laboratory. Whereas arthropod-infecting *Wolbachia* are parasitic symbionts, nematode-infecting *Wolbachia* are obligatory mutualists (Pfarr *et al.*, 2007).

(c) *Strains of* Wolbachia pipientis

For a number of years, nucleotide sequences in the *Wolbachia* surface-protein gene *wsp* were used to characterize genetically distinct strains and to provide a basis for strain classification, even though this single-copy gene is highly variable (Braig *et al.*, 1998; Zhou *et al.*, 1998). Use of *wsp* in the characterization of *Wolbachia* strains was discouraged by the finding that it recombines not infrequently (Jiggins, 2002). Analysis of nucleotide and amino acid sequence variations in *wsp* showed four hypervariable regions separated by regions under strong conservation, and revealed that exchanges occur between strains within and across the arthropod supergroups (Baldo *et al.*, 2005). The use of *wsp* for strain characterization and identification was superseded by multilocus sequence typing (MLST), which involves the sequencing of five conserved housekeeping genes (Section 46.1.2). Typically, the *Wolbachia pipientis* MLST scheme uses fragments of the genes *ftsZ*, *gatB*, *coxA*, *hcpA* and *fbpA* for typing, and makes it possible to relate any new strain to other strains recorded in the Wolbachia MLST Databases (http://pubmlst.org/wolbachia/). Baldo *et al.* (2006b) reported that the MLST system provides an unambiguous tool for strain typing, population genetics, and molecular evolutionary studies, but suggested that *wsp* be retained as an additional strain marker to complement the information from MLST, on the grounds that WSP surface-protein typing is comparable to the antigen-protein typing used for pathogenic bacteria.

The name given to a strain usually consisted of the italicized letter *w* as prefix followed by the non-italicized first three letters of the species name of a nominated arthropod host, the 'reference species'; e.g. *w*Pip for the symbiont strain from *Cx. pipiens*. Wolbachiae with the characteristics of *w*Pip have been isolated from a number of host species (Table 46.17). In some cases, the name of a *Wolbachia* strain was the prefix *w* followed by the first two letters of the name of the locality where the reference species was collected, e.g. *w*Ri for *Drosophila simulans* (Riverside) and *w*No for *D. simulans* (Noumea).

Table 46.17 Classification by strain and supergroup of isolates of *Wolbachia pipientis*, with the names of their host species. For supergroups A and B, emphasis is given to isolates from mosquitoes and *Drosophila* species of the Melanogaster Complex; the selection omits many strains known from other arthropod hosts. The supergroups to which other *Wolbachia* strains have been assigned are: supergroups C and D – hosts, nematodes; supergroup E – hosts, collembolans (springtails); supergroup F – hosts, insects and nematodes; supergroup H – hosts, termites; supergroup I – hosts, fleas; supergroup J – host, filarial nematode; supergroup K – host, phytophagous mite. Supergroup G – host, spiders (refs 10, 14) is no longer accepted. Strain names have been given to the host species listed under supergroups A and B. For supergroup C they apply to the species first listed. For supergroups D to K strain names have been given only seldom or not at all.

Strain	*Host species*	*Refs*
	Supergroup A	
wMel	*Drosophila melanogaster* (*yw67c23*), *Stegomyia polynesiensis*	1, 6
wCof	*Drosophila simulans* (Coffs Harbor)	1, 2
wScu	*Stegomyia pseudoscutellaris*, *St. polynesiensis*, *Stegomyia tongae*	8
wAlbA	*Stegomyia albopicta* (Houston), *Stegomyia pseudoalbopicta*	1, 2, 5, 6
wHa	*D. simulans* (Hawaii), *Drosophila sechellia*	1, 9
wRi	*D. simulans* (Riverside)	1, 2, 9
wSub	*Armigeres subalbatus*	3, 6
	Supergroup B	
wPip	*Culex pipiens*, *Culex quinquefasciatus*, *Culex sitiens*	1, 2, 5, 6
wNo	*D. simulans* (Noumea)	1
wMa	*Drosophila mauritiana*	1, 2
wAlbB	*St. albopicta* (Houston), *St. pseudalbopicta*	1, 2, 6
wCon	*Culex gelidus*, *Cx. sitiens*, *Coquillettidia crassipes*, *Mansonia indiana*, *Stegomyia riversi*	5, 6
wUnif	*Mansonia uniformis*	3, 6
wVul	*Armadillidium vulgare*	2, 9
	Supergroup C	
wDim	*Dirofilaria immitis*, *Dirofilaria repens*	4a, 4c, 4d
wOvo	*Onchocerca volvulus*, *Onchocerca ochengi*	4a, 4c, 4d
	Supergroup D	
wBm	*Brugia malayi*, *Brugia pahangi*	4a, 4c, 4d
	Wuchereria bancrofti	4a, 4c, 4d
	Litomosoides sigmodontis	4a, 4c, 4d
	Supergroup E	
	Mesaphorura macrochaeta, *Folsomia candida*, *Paratullbergia callipygos*	4c, 4d, 7, 11, 12
	Supergroup F	
	Species of *Coptotermes*, *Cryptotermes*, *Heterotermes*, *Hospitalitermes*, *Kalotermes*, *Microtermes*, *Nasutitermes*	4d, 7, 14
	Cimex lectularius, *Oeciacus vicarius*	7
	Columbicola columbae, *Hohorstiella lata*	11
	Opistophthalmus spp.	15
	Mansonella ozzardii, *M. perstans*	4b, 4d, 15, 16
	Supergroup H	
	Zootermopsis angusticollis, *Zootermopsis nevadensis*	10
	Supergroup I	
	Ctenocephalides felis, *Ctenocephalides canis*, *Orchopeas leucopus*	10, 17

Continued

Table 46.17 Continued.

Strain	*Host species*	*Refs*
	Supergroup J	
	Dipetalonema gracile	10, 17
	Supergroup K	
	Bryobia species V	17

Abbreviations: B., Brugia. Cq., Coquillettidia. Cx., Culex. D., Drosophila. Di., Dirofilaria. Ma., Mansonia. St., Stegomyia. Z., Zootermopsis.
References: 1, Zhou *et al.* (1998); 2, Van Meer *et al.* (1999); 3, Ruang-Areerate *et al.* (2003); 4a. Casirhagi *et al.* (2001a); 4b, Casirhagi *et al.* (2001b); 4c, Casirhagi *et al.* (2004); 4d, Casirhagi *et al.* (2005); 5, Kittayapong *et al.* (2000); 6, Dean and Dobson (2004); 7, Rasgon and Scott (2004); 8, Behbahani *et al.* (2005); 9, Jeyaprakash and Hoy (2000); 10, Bordenstein and Rosengaus (2005); 11, Covacin and Barker (2007); 12, Czarnetzki and Tebbe (2004); 13, Goodacre *et al.* (2006); 14, Lo and Evans, (2007); 15, Baldo *et al.* (2007); 16, Keiser *et al.* (2008); 17, Ros *et al.* (2009).

Reference strains are identifiable by their GenBank accession numbers. By 2006, more than 450 different strains of *W. pipientis*, with unique gene sequences, had been deposited in GenBank (Riegler and O'Neill, 2006).

Most, and possibly all, populations of *Cx. pipiens* and *Cx. quinquefasciatus* that are infected with *W. pipientis* are singly infected with *w*Pip. The remarkably large number of crossing types reported from wild populations of *Cx. pipiens* and *Cx. quinquefasciatus* and the complex array of unidirectional and bidirectional incompatibilities that they display (Laven, 1967; Guillemaud *et al.*, 1997; Rasgon and Scott, 2003) appear inconsistent with the restriction of *W. pipientis* from those two species to the single strain *w*Pip (Table 46.17), but that finding has not been overturned. In contrast, almost all populations of *Stegomyia albopicta* are superinfected with two strains of *Wolbachia*, *w*AlbA and *w*AlbB, which are from supergroups A and B, respectively.

In the case of nematode hosts and their symbionts, in which coevolution has occurred, phylogenetic trees indicate a clear separation between symbiont strains (Bandi *et al.*, 1998; Casiraghi *et* al., 2005). This suggests that isolates of symbiont from any host species are of a genetically coherent type, i.e. represent a single strain. Names have been given to a few strains infecting nematodes, including *w*Bm (for strains in *Brugia malayi*), *w*Ovo (strains in *Onchocerca volvulus*) and *w*Dim (strains in *Dirofilaria immitis*; Fenn and Blaxter, 2006). Eventually the practice of naming strains in this manner ended, with only a very few exceptions.

(d) Wolbachia pipientis *groups*

During the early stages of strain classification, named strains of *W. pipientis* were assigned to 'groups', the members of a group sharing a close similarity of *wsp* sequences with those in a nominated reference strain. The name of each group came from the first three letters of the name of its reference strain, with no *w* prefix, e.g. the groups Mel, Sub, Con and Pip. Many groups comprised strains of *Wolbachia* from unrelated hosts. For example, the group Pip included *w*Pip from *Cx. pipiens* as the reference strain, *w*AlbB from *St. albopicta*, *w*Ma from *Drosophila mauritiana*, and further strains from lepidopteran, hymenopteran or homopteran hosts. The group Mel included *w*Mel from *Drosophila melanogaster* as reference strain, *w*Fus from *Aphelinus fuscipennis* (a *Drosophila*-infecting parasitoid), and *w*Scu from three members of the *Stegomyia scutellaris* group (Zhou *et al.*, 1998; Van Meer *et al.*, 1999; Ruang-Areerate *et al.*, 2003; Behbahani *et al.*, 2005).

Sequence analysis of *wsp* from different strains revealed that recombination had taken place between strains of *Wolbachia* from supergroups A and B (Jiggins, 2002). For that reason, groups that had been established from *wsp* sequences were no longer considered valid, and the rank of 'group' is no longer used in the classification of *Wolbachia*.

(e) Wolbachia pipientis *supergroups*

Analysis of *ftsZ* sequences in strains of *W. pipientis* isolated from a number of arthropod but mainly insect hosts placed them in two distinct clusters, A and B (Werren *et al.*, 1995a,b). During the period 2004 to 2006, the MLST method involving sequence analysis of internal fragments of the genes *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* came into use, for both establishing the affinities of different strains (as noted in Subsection 46.9.2.c above), and distinguishing supergroups as being the major phylogenetic divisions (clades) of the genus *Wolbachia* – the use of *wsp* analysis for the same purpose having proved defective, in some instances having led to the wrong conclusions owing to recombination.

Strains were isolated from a wide range of arthropod and filarial nematode taxa, and phylogenetic analyses by MLST eventually grouped the many lineages into a number of major clades, which were ranked as 'supergroups' and identified by the letters A to K (Table 46.17; Figure 46.19). Of those, supergroup G has been discarded. It had been designated for strains of *Wolbachia* present in certain Australian spiders on the strength, very largely, of *wsp* sequences (Rowley *et al.*, 2004; Goodacre *et al.*, 2006), but on reanalysis by MLST the strains were shown to be supergroup A/B recombinants (Baldo and Werren, 2007). Supergroup K should, perhaps, be considered tentative. It was designated for a strain of *Wolbachia* found in a species of phytophagous mite of the genus *Bryonia* (Acari, Phytoseiidae), but the species could not be identified and was given the informal name 'species V' (Ros *et al.*, 2009).

Of strains of *W. pipientis* from 40 host species that were characterized and assigned to supergroups, 35 strains were assigned to supergroups A and B but only five strains to supergroups D, E and F. For the 35 strains of supergroups A and B the MLST primers worked reliably, detecting diversity among *Wolbachia* strains within a host species and identifying closely related strains in different arthropod hosts (Baldo *et al.*, 2006b; Baldo and Werren, 2007).

All *Wolbachia* strains assigned to supergroups A, B, E and H were symbionts of arthropods. The type strain of *W. pipientis* (*w*Pip) fell into supergroup B. The hosts of wolbachiae belonging to supergroups C, D and J were exclusively filarial nematodes. Strains from arachnid hosts have been assigned to supergroups A, B and K, strains from isopod hosts to supergroup B. The known hosts of supergroup F strains are of great variety, and include species of seven genera of termites, two genera of bedbugs, two of bird lice and one of filarial nematodes. Supergroup H was designated for strains from certain termites (Isoptera) (Lo *et al.*, 2002, 2007; Casiraghi *et al.*, 2005; Lo and Evans, 2007). Combined analysis of the three protein-coding genes *glt*A, *ftsZ* and *groEL* gave a well-supported representation of the supergroup designations, agreeing with the earlier findings (Ros *et al.*, 2009) (Figure 46.19). There is no clear definition of the term supergroup; rather, supergroups emerge from continuing cladistic analyses that demonstrate an equivalence of clades. Probably, as more strains of *W. pipientis* are analysed, further supergroups will be identified.

From the analysis of many genes, it was estimated that nematodes diverged from the lineage leading to arthropods and chordates over a billion years ago (Wang *et al.*, 1999), and there is evidence that the symbiosis of *Wolbachia* with filarial nematodes has been stable and species specific for long evolutionary periods (Taylor *et al.*, 2005). The great majority of nematode-infecting strains of *Wolbachia* belong, and exclusively so, to supergroups C, D or J; only the strains that infect two species of *Mansonella* share supergroup F with arthropods (Table 46.17). Pfarr *et al.* (2007) argued that the nematode-infecting strains of supergroups C and D should be treated as distinct species of *Wolbachia*.

(f) Cytoplasmic incompatibility (CI) types within strains

Where a mosquito species is host to a particular strain of *W. pipientis*, populations in geographically separate areas may be incapable of interbreeding when brought together, owing to incompatibility between the different forms of the symbiont strain present in the mosquitoes (Section 46.9.6). Host

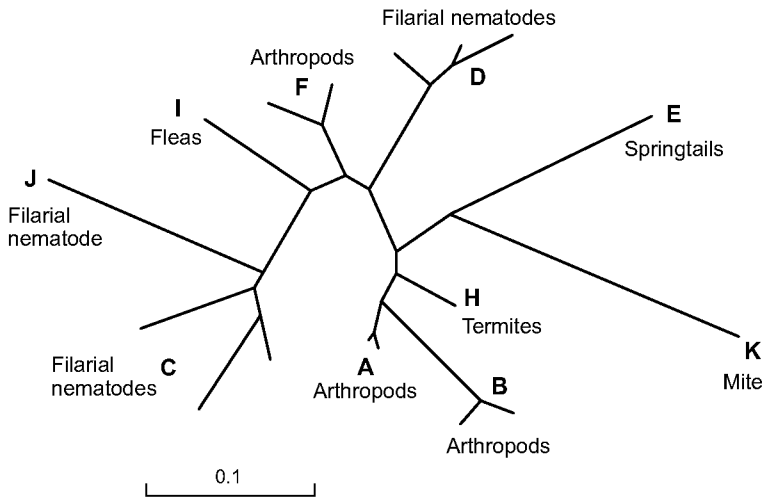


Figure 46.19 Maximum likelihood, unrooted phylogenetic tree of *Wolbachia* concatenated from the sequences of three protein-coding genes (*gltA*, *ftsZ*, *groEL*). (After Ros *et al.*, 2009.) The tree indicates the evolutionary relationships of supergroups A to K, supergroup G being omitted as having no standing. Branches of the lineages represent strains. The scale bar indicates a branch length of 10% likelihood distance. Table 46.17 provides summarized details of the composition of these supergroups.

populations that differ in compatibility are distinguished as 'crossing types', and are said to be of different cytoplasmotypes or cytotypes. The variants of a *W. pipientis* strain that determine crossing type are termed 'cytoplasmic incompatibility types' (CI types).

The forms of *W. pipientis* that infect *Cx. pipiens* and *Cx. quinquefasciatus* are all members of strain *w*Pip. However, geographically separate populations of both of those species are usually of different crossing types, and must therefore be hosts to different *W. pipientis* CI types. Historically, the *Culex* crossing types were given two-letter names adapted from their geographical locations; for example, Ha (Hamburg), Og (Oggelshausen), Pa (Paris), Tu (Tunis), Al (Algiers), De (Delhi), Pe (Penang), Ba, Bakersfield (Laven, 1959, 1967).

46.9.3 The genome

(a) The chromosomal genome

Characteristically, the genomes of bacteria are small; furthermore, obligate, intracellular bacteria usually exhibit gene loss and their genomes are smaller than those of free-living bacteria. Adaptation to the constant environment provided by the cytoplasm of a eukaryotic cell allows these bacteria to reduce their anabolic capabilities if they can absorb metabolic precursors from the host cell (Huigens and Stouthamer, 2003). Within the class Alphabacteria, the known genome sizes of free-living species range from 3.0 to 8.7 mbp, whereas those of obligate intracellular species are below 2.0 mbp. Measured by electrophoresis of purified DNA, the genome sizes of *Wolbachia* strains from two insect and two nematode hosts ranged from 0.95 to 1.66 mbp (Sun *et al.*, 2001).

The *Wolbachia* genome is contained in one double-stranded DNA molecule, which is circular and without 5′ or 3′ ends. Complete genome sequences have been described for three strains of *Wolbachia* from supergroups A, B and D: these are, respectively: (i) *w*Mel from *Drosophila melanogaster* (yw^{67c23}) (comprising 1,267,782 bp) (Wu *et al.*, 2004); (ii) *w*Pip from the colonized Pel strain of *Culex pipiens* (comprising 1,482,355 bp) (Klasson *et al.*, 2008); and (iii) *w*Bm strain from *Brugia malayi* (comprising 1,080,084 bp) (Foster *et al.*, 2005). Both the *w*Pip and *w*Mel genomes contain large amounts

of repetitive DNA, and of DNA corresponding to mobile genetic elements. Repeats of >200 bp make up 14.2% of the genome, and most of the difference in genome size between the *w*Pip and *w*Mel genomes is due to repeated and mobile elements. The suspected mobile elements include insertion sequence (IS) elements and retrotransposons. The *w*Pip genome contains 116 IS elements and eight additional coding sequences similar to transposases. The IS elements of *w*Pip and *w*Mel are dissimilar in composition and distribution. Although even more degraded than the genomes of *Rickettsia* species, the *w*Mel and *w*Bm genomes encode for more metabolic pathways, including those for synthesis of riboflavin, flavin adenine dinucleotide, haem and nucleotides, but they have lost the genes that encode pathways of amino acid biosynthesis. Comparison of the genomes of strains *w*Mel, *w*Pip and *w*Bm reveals a high degree of shuffling. Exceptionally within the class Alphabacteria, all of these *Wolbachia* genomes contain an unusually high number of genes that encode repeats of ankyrin (Wu *et al.*, 2004; Foster *et al.*, 2005; Klasson *et al.*, 2008).

Ankyrins (ANK) are large, internal attachment proteins with an ANK domain, i.e. a repeat motif of 33 amino acids with distinct secondary and tertiary structures. Sixty genes in the *w*Pip genome contain one or more ankyrin repeat domains, compared with 23 genes in *w*Mel and 39 in *w*Ri. In *w*Pip, two of the genes are present in identical duplicates and one in identical triplicates, leaving 56 unique ANK genes. Only 25 of the 60 *ank* genes are homologous with any *w*Mel *ank* gene. A total of 19 of the *w*Pip ANK genes are found within or next to the predicted five prophage regions (Klasson *et al.*, 2008). The possible involvement of ankyrins in cytoplasmic incompatibility is discussed in Section 46.9.6.e.

Genes for a Type IV secretion system (Section 46.4.2), present in many pathogenic bacteria, have been found in *Wolbachia*. Such genes often occur as an operon that consists of several *virB* genes and a *virD* gene. As in other endosymbiotic rickettsial bacteria, *Wolbachia* have lost non-essential genes, including those that encode the pilus-associated proteins that function in other Type IV secretion systems, but the genes satisfying essential requirements have been conserved. In *Wolbachia* these are clustered in two operons. In the strain *w*Ri infecting *Drosophila simulans*, one operon consisted of five *vir* genes (*virB8*, *virB9*, *virB10*, *virB11* and *virD4*) and the downstream *wspB* locus. The other operon was composed of three genes (*virB3*, *virB4* and *virB6*) and included four additional open reading frames (*orf1* to *orf4*). In insect hosts infected with different *Wolbachia* strains, the *vir* genes were polycistronically transcribed (Rancès *et al.*, 2008).

An examination of 37 *Wolbachia* strains from arthropod and nematode hosts showed that the *vir* gene sequence and organization are strictly conserved, and induce phenotypes such as cytoplasmic incompatibility, feminization or oogenesis in different arthropod hosts. In contrast, extensive variation was shown in genomic sequences flanking the *virβ8–D4* operon, possibly reflecting genetic drift (Pichon *et al.*, 2009).

(b) The phage genome – WO phage

Bacteriophages, or phages, are viruses of the order Caudovirales that infect bacteria. Phages may exist as free particles within the host cell or as prophage, when their dsDNA genome is integrated into the host genome. The phages in *Wolbachia* are termed WO phages, and occur both as free particles and as prophage. The genomic structure and other characteristics of WO phages are described in Section 46.9.8.b.

(c) Horizontal transfer and recombination

Examples are known of where 'sections' or greater amounts of the *Wolbachia* genome have been transferred to the chromosomes of their insect hosts. Finding that 44 of 45 genes spaced throughout the *W. pipientis* genome were present in the nuclear genome of specimens of *Drosophila ananassae* that had been rendered aposymbiotic, Hotopp *et al.* (2007) concluded that nearly the entire symbiont genome had been transferred to the fly nuclear genome. Klasson *et al.* (2009) regarded such trans-

fers as recent events, and they surmised that the transferred sequences were on an evolutionary trajectory to degradation and loss.

Natural populations of *Stegomyia aegypti* are not known to harbour wolbachiae. To find whether *St. aegypti* had ever been a natural host, its sequenced genome was examined for genes that could have originated in *W. pipientis*. The *St. aegypti* gene AAEL004181 was found to share *c.* 50% amino acid identity with two genes (WP1348 and WP1346) in strain *w*Pip from *Cx. quinquefasciatus*, probably once a single gene but split by insertion of an IS element. The adjacent *St. aegypti* gene (AAEL004188) showed partial similarity to *w*Pip WP1349, and was inverted compared with the *Wolbachia* genes. Klasson *et al.* (2009) concluded that the extent of sequence identity and the involvement of adjacent sets of genes provide evidence for horizontal gene transfer at an early date.

Genetic studies have indicated that homologous recombination, i.e. the asymmetrical replacing of a sequence in a recipient genome with homologous DNA from a donor genome, is a source of genetic variability among strains of *Wolbachia*. Recombination requires infection of an individual host with two or more strains, a condition known in some arthropods, but not in filarial nematodes, infected with *Wolbachia*.

In a study of recombination in *W. pipientis*, alignments were screened for evidence of recombination within or between fragments of four housekeeping genes (*gltA*, *dnaA*, *ftsZ* and *groEL*) from 22 strains in supergroups A and B isolated from insects of four orders. Significant intragenic recombination was detected only in *gltA*, providing evidence of horizontal DNA transfers between strains of the two supergroups. No intragenic recombination was detected in the other three genes. Intergenic recombination was detected between all possible gene pairs, suggesting either horizontal exchange of a genome portion encompassing several genes or multiple recombination events involving smaller tracts along the genome. The observed pattern was compatible with widespread recombination (Baldo *et al.*, 2006a). A further characterization by MLST of fragments of five genes (*gatB*, *coxA*, *hcpA*, *fbpA* and *ftsZ*) from 37 strains belonging to supergroups A, B, D, E and F was taken by Baldo *et al.* (2006b) to confirm the high degree of recombination in chromosomal genes. However, it had been shown that no recombination occurs between wolbachiae infecting the nematodes of supergroups C and D, whereas it was frequent among supergroup A and B wolbachiae which contain greater amounts of mobile DNA (Wu *et al.*, 2004).

46.9.4 Hosts of *Wolbachia*

(a) Analysis of sampling data

For some years, investigators relied on electron microscopy to reveal the presence of wolbachiae in host cells. Later, the use of molecular genetic techniques facilitated the sampling of possible hosts. Regions of certain *Wolbachia* genes (e.g. *ftsZ*, *wsp*, and the 16S rRNA gene) provided primers for PCR. Estimates of infection rates, which were mostly performed on only one or two individuals per species, suggested that at least 20% of all insect species are infected with *Wolbachia*. More recently, a meta-analysis of published data revealed deficiencies in the earlier studies. The meta-analysis used a beta-binomial model that describes the distribution of infection frequencies, and provides estimates of the pattern of infection rate within species and of the overall infection rate among insects generally. This study produced two important results. (i) The frequency of *Wolbachia* infection within any single host species is, typically, either very high (>90%) or very low (<10%). The earlier studies had under-sampled many species. (ii) An estimated 66% of all insect species are infected with *Wolbachia* (Hilgenboeker *et al.*, 2008).

(b) Mosquito hosts

All the surveys noted here of *W. pipientis* infections in mosquitoes predated the meta-analysis that revealed the limitations of those surveys.

Screening by molecular techniques of 141 mosquito species collected from wild populations in Taiwan, Thailand, Italy, and East and West Africa

revealed infection with *W. pipientis* in 43 species of 13 genera (Table 46.18). A number of mosquito species harboured *W. pipientis* strains of both A and B supergroups. Of 34 *Anopheles* species tested, none was infected. *W. pipientis* has not been recorded from any *Anopheles* species, but two strains were maintained through >30 passages in *An. gambiae* cell lines (Rasgon *et al.*, 2006b).

The findings from most studies of cytoplasmic incompatibility in wild populations of *Culex pipiens* and *Cx. quinquefasciatus* indicated that *W. pipientis* is universally present in those hosts. Further, PCR

Table 46.18 The presence or absence of *Wolbachia pipientis* in species of 26 mosquito genera, collected from natural populations in Taiwan, Thailand, Italy, and East and West Africa. (From the data of Kittayapong *et al.*, 2000; Ricci *et al.*, 2002; Tsai *et al.*, 2004.)

	Number of species		*Number in supergroup*		
Genus	*Tested*	*Infected*	*A*	*B*	n
Anopheles	34	0			705
Aedine genera					
Aedes	2	1	–	1	73
Armigeres	6	4	3	2	245
Christophersiomyia	1	0			10
Dahlia	1	1			3
Danielsia	1	1	1	–	2
Diceromyia	1	0			3
Downsiomyia	3	2	2	–	15
Finlaya	10	1	1	–	50
Fredwardsius	1	0			27
Heizmannia	2	0			13
Neomelaniconion	1	0			30
Ochlerotatus	8	3	–	3	1186
Stegomyia	14	5	3	5	384
Tanakaius	1	0			45
Verrallina	1	0			20
Other Culicine genera					
Coquillettidia	2	2	1	2	8
Culex	35	16	11	6	2959
Culiseta	1	0			1
Hodgesia	1	1	1	–	6
Malaya	1	1	1	1	10
Mansonia	4	2	2	2	96
Mimomyia	1	0			1
Orthopodomyia	2	0			4
Topomyia	1	0			3
Tripteroides	3	2	2	1	57
Uranotaenia	5	3	2	2	35
Totals	141	43	30	25	5991

The presence of *W. pipientis* and the identification of supergroups were determined by DNA extraction, PCR and sequence analysis. For 55 of the species tested, the sample size was <10 specimens. The sample sizes of two species were exceptionally large: *Ochlerotatus caspius*, 1051; *Culex modestus*, 2326.

assay with *wsp* primers detected the presence of *W. pipientis* in Pipiens Complex specimens that originated from 67 locations covering all five zoogeographical regions (Duron *et al.*, 2005). However, a few exceptions have been reported. In samples collected in Johannesburg, South Africa, *W. pipientis* was present in *Cx. quinquefasciatus*, but absent from *Cx. pipiens* or, rarely, present at low frequency (Cornel *et al.*, 2003; Rasgon *et al.*, 2006a).From relatively small samples, infection rates in *Cx. pipiens* in the Upper Rhine Valley, Germany, were 13–75%, and in *Cx. quinquefasciatus* in Cebu City, the Philippines, 17% (Mahilum *et al.*, 2003). The oocytes of two Australian Pipiens Complex species, *Culex australicus* and *Culex globocoxitus*, lacked wolbachiae but contained clumps of virus-like particles (Irving-Bell, 1974, 1977).

Some species of *Stegomyia* are susceptible to infection, notably species of the Scutellaris Group. At one stage, examination of the gonads of 13 species of that group showed wolbachiae to be present in 11 species and absent from two (Beckett *et* al., 1978; Wright and Barr, 1980; Wright and Wang, 1980; Trpis *et al.*, 1981; Meek, 1984). The Scutellaris Group includes over 30 species, which have been further separated into the Albopicta and Scutellaris Subgroups (Huang, 1979a). A majority of those species are native to Oceania, the islands of the south-west Pacific Ocean. Some of these, e.g. *Stegomyia polynesiensis*, are distributed through several island groups, whereas others, such as *Stegomyia cooki*, are known from only one island. *Stegomyia scutellaris* occurs on Indonesian islands and in New Guinea; *Stegomyia katherinensis* is found in northern Australia; and *Stegomyia riversi* occurs in the Ryuku Islands of Japan (Macdonald, 1976).

A number of Scutellaris Group species are vectors of filarial nematodes. In French Polynesia, *St. polynesiensis* is the main host of *Wuchereria bancrofti*, and there the mosquito host is infected with a strain of *Wolbachia* supergroup A, whereas its filarial parasite is infected with a strain of supergroup D (Plichart and Legrand, 2005). *Wolbachia pipientis* has never been found in natural populations or laboratory colonies of *St. aegypti*; however, the *w*AlbB strain could be transfected from *St. albopicta* into *St. aegypti*. Crosses between the transgenic and uninfected *St. aegypti* had the same cytoplasmic incompatibility characteristics as those shown for an aposymbiotic × infected cross of *Cx. pipiens* (see Figure 46.21) (Xi *et al.*, 2005).

Before the concept of cytoplasmic incompatibility in mosquitoes had been developed, Woodhill (1949) described non-reciprocal fertility in crosses between *St. scutellaris* and *St. katherinensis*. The cross kath ♀ × scut ♂ resulted in normal egg numbers but total sterility. During the 1970s and 1980s, crosses and backcrosses undertaken between species from different regions of Oceania showed the four compatibility/incompatibility patterns known in *Cx. pipiens* and *Cx. quinquefasciatus*, and were generally consistent with the concept of cytoplasmic incompatibility (Meek and Macdonald, 1984; Meek, 1988, review).

(c) *Other arthropod hosts*

Insects other than mosquitoes. A high proportion of insect species are infected with *W. pipientis*, mostly of supergroups A or B, but a few of the strains of *W. pipientis* that infect insects have been assigned to other supergroups: (i) to supergroup F – strains from seven genera of termites, two of bedbugs and two of bird lice; (ii) to supergroup H – strains from two species of termite; and (iii) to supergroup I, strains from three species of flea (Table 46.17).

Collembola. *Wolbachia pipientis* of supergroup E has been isolated from three species of Collembola.

Arachnids. *Wolbachia pipientis* has been detected in phytophagous mites of the genera *Tetranychus* and *Bryobia* (family Tetranychidae), and assigned to supergroup B (Johanowicz and Hoy, 1996). One undescribed species of *Bryonia* was assigned to the provisional supergroup K (Ros *et al.*, 2009). Wolbachiae have been reported from a number of families of spiders (Araneae) (Werren and Windsor, 2000). The Australian spiders for which a new supergroup (G) was briefly recognized (Rowley *et al.*, 2004; Goodacre *et al.*, 2006) were shown to be recombinants between strains of supergroups A and B (Baldo and Werren, 2007; Ros *et al.*, 2009).

Wolbachiae of supergroup F were detected in scorpions of the genus *Opistophthalmus* (Baldo *et al.*, 2007).

Crustaceans. Tests on species from a range of crustacean orders revealed infections with *Wolbachia*, but mostly in species of the order Isopoda, and predominantly in terrestrial species (woodlice) of the superfamily Oniscoidea. Only a low percentage of aquatic species carried infections. *Wolbachia* were found also in two species of intertidal Amphipoda (Bouchon *et al.*, 1998; Cordaux *et al.*, 2001) and in two species of the entirely aquatic order Ostracoda (Baltanás *et al.*, 2007); these strains of *Wolbachia* belonged to supergroups A or B.

(d) Nematode hosts

Many species of the family Onchocercidae (superfamily Filarioidea) rely on haematophagous arthropods for biological transmission to their vertebrate definitive hosts. *Wolbachia* has developed endosymbiotic associations with species in seven onchocercid genera. Strains symbiotic with species in six of those genera belong to supergroups C, D or J, all of which include only strains associated with filarial nematode hosts (Table 46.17). Supergroup F strains infect a wide range of arthropods and two species of the nematode genus *Mansonella* (Bandi *et al.*, 1998; Casiraghi *et al.*, 2001a,b, 2004, 2005; Tsai *et al.*, 2007; Ros *et al.*, 2009). Among non-filarioid nematodes, *Wolbachia* has been found only in *Angiostrongylus cantonensis* (superfamily Metastrongyloidea; family Protostrongylidae). This symbiont appeared to be genetically distinct from other strains of *Wolbachia*, and could not be assigned to a supergroup (Tsai *et al.*, 2007).

Comparison between infected and aposymbiotic individuals of a number of species of filarioid nematode showed infections with *Wolbachia* to be beneficial to larval development and to fertility (Taylor *et al.*, 2005). As previously mentioned, although the genome of *Wolbachia* is even more degraded than those of closely related *Rickettsia* species, it has retained more intact metabolic pathways. In the case of *Wolbachia* from *Brugia malayi*, Foster *et al.* (2005) concluded from the genome sequences that the mutualist symbiont contributes essential compounds such as nucleotides, haem and riboflavin to the host nematodes.

(e) Manipulation of arthropod hosts

Wolbachia pipientis manipulates its arthropod hosts by inducing one or other of four reproductive abnormalities: (i) parthenogenesis – development of an individual from a female gamete without fertilization by a male gamete; (ii) feminization – conversion of genomic males into functional phenotypic females; (iii) male killing – the killing of male embryos; or (iv) cytoplasmic incompatibility – developmental arrest of the offspring of uninfected females mated to infected males. All of these effects on the host phenotype confer a reproductive advantage on infected host females which, in turn, results in spread of the maternally inherited symbiont through uninfected host populations. In situations where not all populations of a species are infected with *Wolbachia*, infected females or infected strains gain advantage and the symbiosis is mutualistic, but it is not advantageous to the species as a whole under conditions of interspecific competition.

Females of strains or laboratory colonies of *Cx. pipiens* infected with *W. pipientis* have a fitness advantage in terms of fertility over uninfected females, but are not known to gain or lose fitness in terms of fecundity or survival. Infection with *Wolbachia* in Californian strains of *Cx. pipiens* and *Cx. quinquefasciatus* that developed under field conditions had no significant effect on female fecundity, but the prediction from modelling that infection with *Wolbachia* should spread to fixation (an infection in stable equilibrium) was supported by sampling in the field (Rasgon and Scott, 2003).

In laboratory populations of *St. albopicta*, infection with *Wolbachia* increased the longevity of females but not of males. Infection also increased the fecundity of females, which genetic studies showed resulted from the infection as such and not

from selective forces acting on the mosquitoes. No differences in longevity or fecundity were observed between singly infected and superinfected females. Modelling revealed that one result of the enhanced fecundity was an increase in the rate at which an infected population could invade an uninfected population (Dobson *et al.*, 2002a, 2004). Infection is essential for the symbiont and provides reproductive advantage for the host, and so it is mutualistic. The fact that all wild populations of *St. albopicta* are infected with *W. pipientis* is consistent with this finding.

46.9.5 Distribution in gonadal and somatic tissues of mosquitoes

Wolbachia pipientis are pleiomorphic bacteria, usually rod-shaped or coccoid, averaging 0.8–1.5 μm in length within the range 0.25–1.8 μm. Their whole existence is intracellular and within the cytoplasmic compartment of the host cell, where each bacterium is separately enclosed within a vacuole thought to be formed from the host's cell membrane. As with other intracellular bacteria, a narrow periplasmic space separates the vacuolar membrane from the symbiont's cell wall (Figure 46.20B). *Wolbachia* cells grow and multiply by binary fission within the vacuoles (Yen and Barr, 1974; Wright *et al.*, 1978).

Examination by electron microscopy of the gonads of *Wolbachia*-infected mosquitoes shows the bacteria to be present in male and female germ-line cells. In the earliest stage of formation of an ovarian follicle, three successive divisions of a primordial germ cell lead to the appearance of a cluster of eight cystocytes. Because cytokinesis is incomplete, the cystocytes are interconnected to one another by intercellular bridges. One cystocyte differentiates as an oocyte, and the other seven become nurse cells (Volume 1, Section 19.1). In *St. cooki* and *Culex tigripes*, at least, the intercellular bridges are wide enough to allow the passage of wolbachiae and mitochondria from one cystocyte to another. In

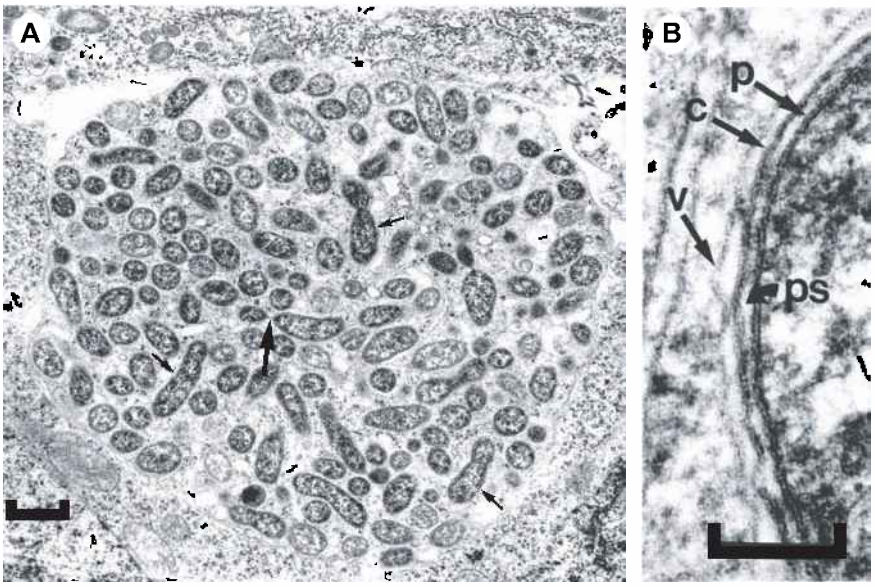


Figure 46.20 *Wolbachia pipientis* in the gonads of pupae or teneral adults of *Culex pipiens*. (From Wright *et al.*, 1978.) **A.** Section through a primary spermatocyte heavily infected with *W. pipientis*. Elongated symbionts (small arrows) are at an early stage of cell division. Large arrow: strand connecting two daughter cells. Bar = 1.0 μm. **B.** Detail of a *W. pipientis* cell, showing the cell wall and plasma membrane separated by a periplasmic space, and the surrounding vacuole membrane of host origin. **c**, cell wall; **p**, plasma membrane; **ps**, periplasmic space; **v**, host-originated vacuole membrane. Bar = 100 nm.

differentiated ovarian follicles, wolbachiae are present in the nurse cells and oocytes (Wright and Barr, 1980; Ndiaye *et al.*, 1995).

In infected testes, wolbachiae are present within spermatocytes (Figure 46.20A) but not within spermatozoa. Characteristically, during spermatogenesis, when spermatozoa cleave from spermatids and become functional cells, residual bodies are left behind and degenerate. In species of *Culex* and *Stegomyia*, during the maturation phase of spermatogenesis, all wolbachiae were contained within the residual bodies and none remained within the spermatozoa (Yen and Barr, 1974; Ndiaye and Mattei, 1993; Ndiaye *et al.*, 1995). The failure of karyogamy that is observed when infected males have mated with uninfected females and their spermatozoa have entered the oocytes indicates that the spermatozoa have been modified, supposedly by imprinting during spermatogenesis. In mosquitoes, as in other hosts of *W. pipientis*, the mode of transmission is transovarian. Infection of the testes is a dead end with regard to transmission, but is essential for the expression of cytoplasmic compatibility or incompatibility.

In recently laid eggs of *St. polynesiensis* containing very young embryos, wolbachiae were distributed in a thin outer layer of cytoplasm adjacent to the endochorion, and also in cytoplasm beside the micropyle. At 48 h, wolbachiae were present in the pole cells but not in other embryonic tissues (Wright and Barr, 1981). In young embryos of *Cx. pipiens*, wolbachiae were sparsely distributed throughout the cytoplasm, but a mass of wolbachiae was present at the anterior pole, just under the micropyle, where they were in close proximity to sperm, and many were located at the posterior pole also, where the pole cells had formed. In developing embryos, the wolbachiae became restricted to the pole cells and later to the gonads. In third- and fourth-instar larvae, the numbers of wolbachiae in the gonads increased rapidly (Yen and Barr, 1974; Rasgon and Scott, 2003).

Within maturing ovarian follicles of *Cx. pipiens*, a proportion of the oocytes and nurse cells were seen to be degenerating. Those ovarian cells contained both healthy-looking wolbachiae and, not infrequently, wolbachiae that appeared swollen and that contained pycnotic regions. The swollen symbionts appeared to be lysing, and often contained particles with the size and structural characteristics of bacteriophage (Wright and Barr, 1980).

Wolbachia pipientis has only very rarely been found by electron microscopy in non-germ-line cells of mosquitoes. It was observed in small numbers of Malpighian tubule cells in *Cx. pipiens* (Yen and Barr, 1974), in cells of the ovarian follicular epithelium in *St. albopicta* (Wright and Wang, 1980), and in salivary gland cells of *Armigeres subalbatus* (Chen *et al.*, 2005). Molecular techniques revealed the presence of *W. pipientis* in a number of somatic tissues of larval and adult *St. albopicta* and *Ar. subalbatus*, including different regions of the alimentary canal, the Malpighian tubules, salivary glands and tracheae. Responses from somatic tissues were weaker than those from the ovaries (Tsai *et al.*, 2004). Wolbachiae were found in heads, thoracic muscle, midgut and Malpighian tubules of adult *Cx. pipiens* and *St. albopicta*. Additionally, they were present in the wings of *Cx. pipiens* and in haemolymph of *St. albopicta* (Dobson *et al.*, 1999).

It has not been possible to grow *W. pipientis* in axenic culture; propagation requires the presence of eukaryotic host cells. A continuous cell line (Aa23) was established with embryonic cells from a strain of *St. albopicta* that was superinfected, but several months later only a single strain of the symbiont was present within the cultured cells (O'Neill *et al.*, 1997). Cells from the *St. albopicta* Aa23 line could maintain strains of *W. pipientis* isolated from *Cx. pipiens*, *D. simulans* and the lepidopteran *Cadra cautella* (Dobson *et al.*, 2002b).

Descriptions of mosquito spermatogenesis, oogenesis, embryogenesis and embryonic development can be found in Volume 1.

46.9.6 Cytoplasmic incompatibility

Defining cytoplasmic incompatibility as 'a functional incompatibility between a maternally inherited cytoplasmic milieu and an introduced factor of male origin', Fine (1978) commented on

its widespread occurrence in nature. Forms of cytoplasmic incompatibility included pollen sterility of maize and other grains, maternally inherited sex-ratio and male-sterility factors in several species of *Drosophila*, and cytoplasmically determined infertility in mosquitoes. In every case, the cause of the incompatibility was a self-replicating extrachromosomal factor, which in several cases was a symbiotic organism – spirochaete, mycoplasma-like organism or rickettsia. Among mosquitoes, cytoplasmic incompatibility has been investigated most intensively in *Culex pipiens* and *Stegomyia albopicta*. Laboratory and field studies with *Drosophila simulans* have provided valuable information on host–symbiont interactions.

In mosquitoes infected with *W. pipientis*, cytoplasmic incompatibility is observed as a failure to complete embryonic development in eggs laid following crosses between certain populations. In a number of species that are subject to infection with *W. pipientis*, crosses between different populations may be fertile, infertile in one direction only, or infertile in both directions. As an example of unidirectional infertility, crosses between infected populations of *Cx. pipiens* from Oggelshausen in southern Germany and Hamburg in northern Germany were fertile in the direction Ha♀ × Og♂ but infertile in the direction Og♀ × Ha♂ (Laven, 1957b). Because of the mechanism of cytoplasmic incompatibility, host females infected with *W. pipientis* have a fitness advantage over uninfected females, and tend to replace uninfected females in any mixed population.

(a) *Cytoplasmic incompatibility in* Culex pipiens *and* Cx. quinquefasciatus

Crossing experiments between many strains of *Cx. pipiens* have led to the identification of a number of 'crossing types', which when outcrossed showed four different patterns of compatibility and incompatibility (Yen and Barr, 1973; Magnin *et al.*, 1987; O'Neill and Paterson, 1992).

(i) Bidirectional compatibility. Crosses in both directions are fertile.

(ii) Unidirectional compatibility (or incompatibility). Crosses are fertile in one direction only, as described above between populations of *Cx. pipiens* from Oggelshausen and Hamburg in Germany.

(iii) Bidirectional incompatibility. Crosses in both directions are sterile (Figure 46.21A).

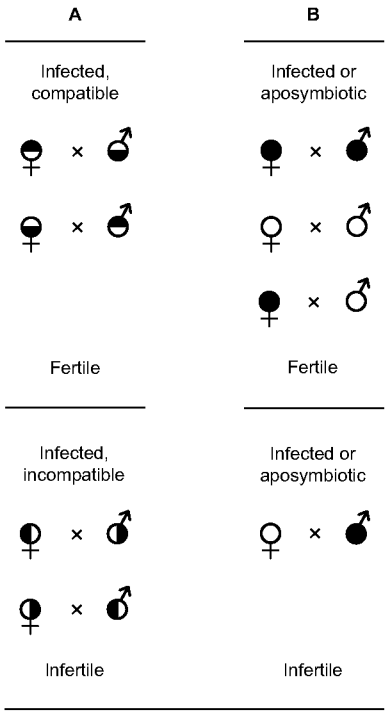


Figure 46.21 Expressions of cytoplasmic compatibility and incompatibility. (Redrawn from Beard *et al.*, 1993.) **A.** In *Culex pipiens*. Crosses in both directions between populations of different crossing type (e.g. ♀, ♂ and ♀, ♂). *Above*. The crossing types of the two populations are bidirectionally **compatible**, so the crosses are fertile in both directions. *Below*. The crossing types of the two populations are bidirectionally **incompatible**, so the crosses are infertile in both directions. **B.** In *Cx. pipiens* and *Drosophila simulans*. Crosses involving two populations, of which one is infected with *W. pipientis* (♀, ♂) and the other is aposymbiotic (♀, ♂). The result is unidirectional incompatibility. *Above*. The within-infected-population cross is fertile in both directions (only one direction is shown). The between-populations cross in which the aposymbiotic partner is male is fertile. *Below*. The between-populations cross in which the aposymbiotic partner is female is infertile.

(iv) Partial compatibility. Embryonic development starts in a high percentage of eggs, and the hatch rate ranges from >1% upwards.

In the incompatible crosses insemination was normal. Further, the eggs were penetrated by sperm, embryonic development proceeded as far as segmentation, and organ formation occurred in a high percentage of eggs; however, very few hatched. Outcrossing experiments revealed a key role of the female lineage. After 50 generations of continuously backcrossing *Cx. pipiens* Oggelshausen females to Hamburg males, the chromosomes of the original Ha♀ parent had been replaced with chromosomes of the Og♂ parent, but even so both male and female descendants possessed the crossing type of the original maternal strain. Laven (1957b) concluded that inheritance of crossing type was controlled by a factor in the cytoplasm, and that the phenomenon was one of cytoplasmic incompatibility.

The crossing characteristics of populations of *Cx. pipiens* and of *Cx. quinquefasciatus* differ in different geographical regions. Laven (1959) reported compatibility and unidirectional and bidirectional incompatibilities between strains of *Cx. pipiens* from 13 locations in Europe. In contrast, strains of *Cx. quinquefasciatus* from 19 locations in South-east Asia were fully compatible in both directions (Thomas, 1971). Each of 11 widely separated Australian populations of *Cx. quinquefasciatus* had a distinct cytoplasmotype. Two populations that were studied in greater detail were found to include at least two segregating crossing types. There was no significant relationship between geographical distance between populations and similarity of crossing type (O'Neill and Paterson, 1992).

On a different geographical scale, crosses between eight strains of *Cx. pipiens* collected within 100 km of Montpellier in southern France showed a variety of unidirectional, bidirectional and partial incompatibilities. None of the eight strains displayed exactly the same crossing relationships as any other. One population included individuals of more than one crossing type, and crossing types were shown to change with time (Magnin *et al.*, 1987). During 1979 and 1980, crossing-type polymorphism was found among populations of *Cx. quinquefasciatus* in the Los Angeles area, at *c.* 34° N (Barr, 1982). However, studies of 14 Californian populations of *Cx. pipiens* and *Cx. quinquefasciatus* during 1999 and 2000, over the latitudinal range 33° 43′ N to 40° 27′ N, revealed only one crossing type (Rasgon and Scott, 2003).

(b) Role of Wolbachia pipientis

Cytoplasmic incompatibility was shown to be caused by the presence of *Wolbachia pipientis* within germ cells. Symbiont-free eggs could be produced by exposing *Cx. pipiens* larvae to the antibiotic tetracycline. Crosses between infected males and aposymbiotic females were sterile, but crosses between aposymbiotic males and either aposymbiotic or infected females were fertile (Figure 46.21B). A cross between a male and a female containing symbionts of different strains resulted in the death of the embryos. Thus, *W. pipientis* was the cause of incompatibility, and cytoplasmotype was determined by the infective agent (Yen and Barr, 1973; Yen, 1975).

A study was undertaken with ten laboratory strains of *Cx. pipiens*, from different geographical regions, which in crosses expressed three types of compatibility: compatible; unidirectionally incompatible; and bidirectionally incompatible. Analysis of DNA sequences from the *ftsZ* gene of *W. pipientis* present in the mosquitoes produced no evidence that any of them was infected by more than a single strain of the symbiont (Guillemaud *et al.*, 1997). In California, 14 strains of *Cx. pipiens* and *Cx. quinquefasciatus* situated between 33° 43′ N and 40° 27′ N all contained only the strain *w*Pip, of group Pip and supergroup B (Rasgon and Scott, 2003). Superinfection (infection with two strains) has not been reported from any population of *Cx. pipiens* or *Cx. quinquefasciatus*.

(c) Mechanism of incompatibility in Drosophila simulans

The most detailed cytological investigations into the mechanism of cytoplasmic incompatibility have

been on *Drosophila simulans*. After incompatible strains had cross-mated, embryogenesis progressed to varying degrees of completion in different proportions of the progeny: (i) arrested during early mitoses, 46.4%; (ii) ending at the syncytial blastoderm stage, 5.8%; (iii) arrested shortly before hatching, 43.0%; and (iv) progressing to completion and hatching, 4.8% (Callaini *et al.*, 1996).

Immunofluorescence was used to show the location of *Wolbachia* before fertilization and during early embryogenesis in *D. simulans*. Before fertilization, wolbachiae were distributed throughout the ooplasm, but during the early cleavage stages they increasingly associated with the cleavage nuclei. During pole cell formation they became localized between the pole cell nuclei and the posterior end of the egg, and in the region of the centrosome. By the 11th mitotic cycle, most wolbachiae were near blastoderm nuclei and associated with centrosomal microtubules. Kose and Karr (1995) concluded that in *D. simulans* the wolbachiae interact specifically with the microtubule-based cytoskeleton, and that these interactions determine their locations during the early stages of embryogenesis.

During the first mitotic division of *Drosophila* zygotes, the maternal and paternal chromosomes come together at the metaphase plate of the spindle, but instead of mingling, as in most animal cells, they progress to anaphase as two widely separated sets and mingle during telophase. In the case of incompatible crosses of *D. simulans*, during the first mitosis of zygotes only the female chromosomes were clearly visible at the end of prophase. The timing of paternal chromatin condensation was delayed. When condensed maternal chromosomes moved to the equatorial plane of the first metaphase spindle, the paternal chromosomes remained uncondensed and entangled in chromatin fibres. In cases where development continued, errors in paternal chromatin inheritance led to the formation of embryos with aneuploid or haploid nuclei. It appeared that the paternal chromosomes had been modified by wolbachiae during spermatogenesis (Callaini *et al.*, 1997).

That conclusion led to the hypothesis that the modification induced in sperm by *W. pipientis* during spermatogenesis is counteracted, or 'rescued', in sperm that enter the cytoplasm of oocytes laid by females infected with the same CI type. Modification (mod) renders sperm incompetent, and results in the failure of karyogamy when such sperm have entered oocytes. Rescue (resc) restores sperm competence, and allows fertilization and normal embryonic development. The hypothesis is most simply described for crosses between infected and uninfected lines of *D. simulans*. (i) First, consider matings by infected males with either uninfected or infected females. When sperm from infected males enter the oocytes of uninfected females, the ooplasm cannot counteract the imprinted factor,* the sperm are not rescued, and the cross is sterile (Figure 46.21B, below). In contrast, when such sperm enter the oocytes of infected females, they are rescued by a factor in the ooplasm and the cross is fertile (Figure 46.21B, above). (ii) When uninfected males mate, their sperm are unmodified and are able to fertilize the oocytes of both uninfected and infected females (Figure 46.21B, above). Expressed in terms of host fitness, the eggs laid by uninfected females that were inseminated with modified sperm are unviable and die, whereas the eggs laid by infected females are viable regardless of whether the male parent was infected or not. By rendering uninfected females less fit, *W. pipientis* increases the relative fitness of infected females, giving them a reproductive advantage over uninfected females.

* **Genomic imprinting.** The specific pattern of methyl groups in germ cell DNA is responsible for the phenomenon of imprinting, which describes a difference in behaviour between the alleles inherited from each parent. The pattern of methylation of germ cell DNA is established during gametogenesis: in males during spermatogenesis and in females during oogenesis. Methylation is usually associated with inactivation of the gene, and at fertilization paternal and maternal alleles may have different patterns of methylation. When genes are differentially imprinted, survival of the embryo might require that the functional allele is provided by the parent with the unmethylated allele. In a heterozygous cross in which the allele of one parent has an inactivating mutation, the embryo will survive if the wild-type allele comes from the father, but will die if it comes from the mother (Lewin, 2008).

Some strains of *Drosophila* neither modify nor rescue sperm. Investigators designated two variants for *W. pipientis* that infect *Drosophila*: (i) $mod^{+}resc^{+}$ variants are capable of modifying male germ cells during spermatogenesis and of rescuing imprinted (modified) sperm within oocytes; (ii) $mod^{-}resc^{-}$ variants are incapable of either modifying sperm or rescuing the modification in eggs. Both variants have been found in wild populations of *D. simulans* (Clancy and Hoffmann, 1996; Merçot and Poinsot, 1998a). A third variant that does not modify sperm but can rescue imprinted sperm ($mod^{-}resc^{+}$) was detected in a population of *D. simulans* on Mt Kilimanjaro, Tanzania (Bourtzis *et al.*, 1998; Merçot and Poinsot, 1998b).

(d) Mechanism of incompatibility in Culex *mosquitoes*

Observations on the progeny from crosses between male and female *Culex pipiens* that were infected by incompatible strains of *W. pipientis*, or in which one parent was infected and the other aposymbiotic, have thrown light on the effects of *W. pipientis* on embryonic development. The course of embryonic development in progeny from such crosses could be assigned to one or other of three categories.

(i) Embryonic development was arrested at a very early stage, with no evidence of cell division.
(ii) Embryonic development was sufficient for organized tissues to form, e.g. stemmata, but there was no extensive differentiation.
(iii) Embryonic development progressed to an advanced stage, but the embryos had severe structural abnormalities.

When incompatible crosses were between infected males and uninfected females, embryonic development was always of category (i). In contrast, when both male and female parents in an incompatible cross were infected, all three classes of embryonic development could be discerned among their progeny (Duron and Weill, 2006).

Investigation of the development of unviable embryos revealed that female meiosis had been completed, and that sperm had penetrated and migrated to the centre of the egg, but that the male and female pronuclei had not fused. Despite this, the haploid female pronucleus had divided, cleavage energids had formed, and in some 75% of eggs a blastoderm had formed and development had proceeded to histological differentiation or beyond. Use of eye-colour mutations, which are visible in developing embryos, confirmed that the sperm contributed no chromosomes to the nuclei of unviable embryos (Jost, 1970a,b).

The role of sperm in activation of early developmental events was investigated by irradiating the male parents. In the compatible *C. pipiens* Hamburg × Hamburg cross, irradiation of males with 5–8 kR led to death of the eggs during the early cleavage stages, yet in the incompatible Hamburg ♀ × Paris ♂ cross, prior irradiation of the males with similar dosages did not affect the number of embryos developing to the blastoderm stage and beyond. The blastoderm nuclei of inviable embryos contained only the haploid number of chromosomes, and predominantly contained a quantity of DNA equal to that in one haploid spermatid nucleus (= C). It appeared that in incompatible crosses not only did penetration of the egg by sperm stimulate the completion of female meiosis, but some factor also stimulated development to the blastoderm stage and beyond in the absence of karyogamy (Jost 1970a, 1971, 1972). As the non-viable embryos that developed from an incompatible cross were haploid, their failure to develop normally may have been due to recessive lethal factors which are expressed only in the hemizygous condition.

When an incompatible cross was made between wild-type males of *Cx. pipiens* and females heterozygous for the dominant mutation Kuf, the few viable adults that had developed parthenogenetically included all possible genotypes: +/+, *Kuf*/+ and *Kuf*/*Kuf*. This recombination of genes suggested to Laven (1957b) that some at least of the progeny had resulted from fusion of the products of female meiosis, i.e. fusion of the female pronucleus with a polar body, or fusion of two polar bodies – a phenomenon known as automictic or meiotic parthenogenesis. Analysis of another incompatible cross involving six marker genes revealed that the four products of meiosis were not equally used in

the formation of parthenogenetic females. Possibly, therefore, the few viable progeny had developed from a diploid nucleus that originated by fusion of the female pronucleus with its sister polar body (Jost, 1970b).

The identity of the mosquito strain, and hence of the strain of *W. pipientis*, affected the proportion of embryos that developed. For example, with *Cx. pipiens*, the proportion of eggs of category (iii), i.e. containing advanced-stage embryos, was 19.3% from a ♀ Slab × ♂ Tunis cross and 53% from a ♀ Slab × ♂ MaClo cross ($p < 0.01$). This was taken to implicate male factors in the embryo's capacity to reach advanced stages. In the incompatible cross MaClo × Istanbul, embryos that were fully formed had maternal chromosomes only, as shown by genetic markers specific for each parent. But, if one parent, whether male or female, was aposymbiotic, the embryos had both male and female chromosomes. Any partly developed embryos from a ♀ Slab × ♂ Tunis cross possessed both male and female chromosomes (Duron and Weill, 2006).

It appears that the wolbachiae transform male gametes in such a way that, although the cellular fusion of male and female gametes can proceed, the interaction between the male and female pronuclei within the ooplasm fails. In other words, syngamy is unaffected but karyogamy is abortive. Any embryos that form are haploid and therefore non-viable, so the females lay infertile eggs. Spermatozoa produced by infected males contain no visible remnant of the symbiont, but their effect on aposymbiotic oocytes indicates that they are modified in some way. As with *Drosophila* (Section 46.9.6.c), it is thought that wolbachiae present in male germ cells of mosquitoes leave an 'imprint' on the spermatozoa that develop from them. During mosquito ovulation, small numbers of sperm pass though the micropyle of an oocyte and into its cytoplasm. If the parents are of compatible cytoplasmotypes, fertilization proceeds normally and embryogenesis starts. If sperm from infected fathers enter oocytes that are uninfected, or from a non-compatible mother, fertilization does not proceed normally and embryogenesis is blocked. The first contact of the sperm is with the oocyte cytoplasm, and it is the nature of that interaction that determines whether or not the male and female pronuclei can fuse (Yen and Barr, 1973, 1974). (The normal processes of fertilization in mosquitoes are described in Volume 1, Section 2.1.1.)

The model of cytoplasmic incompatibility in *Drosophila* (Section 46.9.6.c) has been adapted to account also for the unidirectional and bidirectional incompatibilities that commonly occur between allopatric populations of *Cx. pipiens* and *Cx. quinquefasciatus*. Consider two notional strains, A and B, that are unidirectionally incompatible. Strain B ooplasm is able to rescue sperm modified by strain A, but strain A ooplasm is unable to rescue sperm modified by strain B (Table 46.19C). In the case of two bidirectionally incompatible strains, neither possesses ooplasm able to rescue sperm modified by the other (Table 46.19D).

Where a strain is superinfected (as in *St. albopicta*), it is supposed that its males produce sperm modified by two independently acting molecules, and that its females produce oocytes containing rescue molecules for both. In crosses between superinfected and appropriate singly infected strains, oocytes laid by singly infected females can rescue only one of the modifications of superinfected sperm, so their oocytes fail to develop. In contrast, oocytes laid by superinfected females can rescue the sperm of both singly and superinfected males. The outcome is unidirectional incompatibility (Subsection 46.9.6.f below).

(e) Genetic control of cytotype by strains of w*Pip*

Crosses between populations of Pipiens Complex species have revealed the existence of many crossing types, in each case induced by the *Wolbachia* strain *w*Pip. Several research teams have tried to establish a basis for cytotype determination by genetic variants of *w*Pip, working with many strains of *W. pipientis*.

Greatest attention has been paid to the *ank* genes that are associated with WO (*Wolbachia*) phage and that express ankyrins (ANK) (Section 46.9.3.a). Ankyrins are intracellular attachment proteins that mediate protein–protein interactions of many

Table 46.19 Outcomes of crosses between four notional mosquito strains that are uninfected (U), or singly infected with *Wolbachia pipientis* of different cytotypes (A or B), or that are superinfected with both of the cytotypes (A and B).

Males			*Females*			
Mosquito strain	*Status*	*Sperm*	*Mosquito strain*	*Status*	*Ooplasm*	*Outcome of cross*
A. Within strain crosses						
U	Uninfected	mod^{-}	U	Uninfected	$resc^{-}$	Fertile
A	Infected	mod^{+A}	A	Infected	$resc^{+A}$	Fertile
B	Infected	mod^{+B}	B	Infected	$resc^{+B}$	Fertile
AB	Superinfected	mod^{+AB}	AB	Superinfected	$resc^{+AB}$	Fertile
B. Infected × uninfected crosses						
A	Infected	mod^{+A}	U	Uninfected	$resc^{-}$	Sterile
U	Uninfected	mod^{-}	A	Infected	$resc^{+A}$	Fertile
C. Unidirectionally incompatible crosses						
A	Infected	mod^{+A}	B	Infected	$resc^{+B}$	Fertile
B	Infected	mod^{-B}	A	Infected	$resc^{+A}$	Sterile
D. Superinfected × uninfected or two singly infected and unidirectionally incompatible strains						
AB	Superinfected	mod^{+AB}	U	Uninfected	$resc^{-}$	Sterile
U	Uninfected	mod^{-}	AB	Superinfected	$resc^{+AB}$	Fertile
AB	Superinfected	mod^{+AB}	A	Infected	$resc^{+A}$	Sterile
A	Infected	mod^{+A}	AB	Superinfected	$resc^{+AB}$	Fertile
AB	Superinfected	mod^{+AB}	B	Infected	$resc^{+B}$	Sterile
B	Infected	mod^{+B}	AB	Superinfected	$resc^{+AB}$	Fertile

In **C**, the pattern of unidirectional incompatibility between the notional strains A and B resembles that between two German strains of *Culex pipiens*, namely Oggleshausen (= A) and Hamburg (= B), which were fertile in the direction Og♂ × Ha♀, but infertile in the direction Ha♂ × Og♀ (Section 46.9.6).
In **D**, the patterns of incompatibility between the superinfected strain and the uninfected or singly infected strains resemble those obtained with strains of *Stegomyia albopicta* in the laboratory (Section 46.9.6.f).
mod, modified; resc, rescued.

types, including attachment of proteins to the cytoskeleton. Investigations into the possible involvement of ankyrins in sperm modification and rescue yielded some results that supported that possibility and others that did not. The *w*Mel strain of *W. pipientis*, which is characterized as $mod^{+}/resc^{+}$, contained a gene for ANK that was absent from the closely related strain *w*Au, which is $mod^{-}/resc^{-}$ (Iturbe-Ormaetxe *et al.*, 2005). Further, sequences of *ank* are present in the gene *pk2*, which is located within prophage-like regions of the *W. pipientis* genome. The host-specific expression of *pK2* in males and females of Pel, Bei and other strains was consistent with its possible involvement in sperm modification in males and rescue in females (Sinkins *et al.*, 2005). Not supportive was the result of a search for sequence variability in the 58 *ank* genes, which found only five that were polymorphic in the genomes of incompatible *W. pipientis* variants; none correlated with the patterns of cytoplasmic incompatibility obtained with 15 mosquito strains infected with 14 *W. pipientis* variants (Duron *et al.*, 2006a, 2007a).

Two other factors that were investigated to the same end showed no apparent involvement in cytotype determination: (i) the locus *orf7* in WO phage (Sanogo *et al.*, 2005; Sanogo and Dobson, 2006); and (ii) *Tr1*, also in WO phages, an

apparently functional transposable element of the insertion sequence-5 (IS5) family that can be used to discriminate between *w*Pip variants (Duron *et al.*, 2005).

Despite the complexity of crossing types, no polymorphism of the *w*Pip strain has been found that might account for cytoplasmic incompatibility. Walker *et al.* (2007) considered it plausible, and even probable, that the genetic basis of the many crossing types involves multiple *Wolbachia* genes and interaction between *Wolbachia* variants and the mosquito genome.

(f) *Cytoplasmic incompatibility in* Stegomyia albopicta

Many species of the Scutellaris Group of *Stegomyia* are infected with *W. pipientis*. All natural populations of *St. albopicta*, a member of the Albopicta Subgroup, are infected, and almost all are superinfected, containing symbionts of both strain *w*AlbA (supergroup A) and strain *w*AlbB (supergroup B). Molecular screening of 12 *St. albopicta* strains from geographically dispersed locations revealed superinfection in ten populations. Only the populations in two oceanic islands, Mauritius, and Samui Island in the Gulf of Thailand, were singly infected and in both cases with *w*AlbA (Sinkins *et al.*, 1995; Sinkins, 2004). Populations of *St. albopicta* throughout Thailand were superinfected, but not all individuals were superinfected. Of 320 *Wolbachia*-positive individuals from different regions of Thailand, 97.5% were superinfected. The transmission efficiencies were 96.7% for the supergroup A strains, and 99.6% for the supergroup B strains. A population in Chachoengasao Province, Thailand, sampled by landing catches during a 6 month period, showed 100% infection. The extent of superinfection with *Wolbachia* of supergroups A and B was 99.4%; the remaining 0.96% were singly infected with a supergroup A strain (Kittayapong *et al.*, 2002a,b).

When singly infected male *St. albopicta* mate with uninfected females, the eggs show developmental arrest and the cross is infertile. But, when singly infected females mate with uninfected males, the cross is fertile (Figure 46.22). Expressed in another way, incompatibility is observed when the male host harbours an infection type that is not present in its female mate. When superinfected males mate with uninfected or singly infected females, the crosses are infertile. In contrast, when superinfected females mate with uninfected or singly infected males, the crosses are fertile (Dobson *et al.*, 2001).

A high degree of cytoplasmic incompatibility (mean egg hatch only 0.19–0.40%) was obtained when wild-caught, superinfected adult male *St. albopicta* from two populations (CH and KL) were mated with females of laboratory strains that were either uninfected (UJU) or singly infected (KOH)

Female \ Male	U	A	B	AB
U	$+^{U}$	–	–	–
A	$+^{A}$	$+^{A}$	–	–
B	$+^{B}$	–	$+^{B}$	–
AB	$+^{AB}$	$+^{AB}$	$+^{AB}$	$+^{AB}$

Figure 46.22 A schematic diagram showing the crossing compatibilities between *Stegomyia albopicta* strains of four compatibility types: (i) U, uninfected; (ii or iii) A or B, singly infected with *Wolbachia pipientis* of strain A or B; (iv) AB, superinfected. Strains A and B are incompatible when in singly infected hosts, but can occur together in a superinfected strain. (After Dobson *et al.*, 2001.) Within the boxes: +, compatible cross; –, incompatible cross; superscript letters indicate the cytotype of the offspring of compatible crosses. Incompatibility is observed when the male host harbours a strain of *W. pipientis* that is incompatible with the strain in its female mate. The singly infected females have a reproductive advantage over the uninfected females because they are compatible with both uninfected males and with males of the same cytotype. Superinfected females have an advantage over uninfected and singly infected females because they are compatible with uninfected, singly infected and superinfected males.

(Table 46.20). In contrast, mean egg hatches of 88.6–88.9% were obtained when the superinfected wild males mated with females from a different laboratory colony (KLPP) that were superinfected with the same *Wolbachia* strains. Similar results were obtained when wild-collected eggs from the same two natural populations were reared, and the F_1 males were mated with uninfected or singly infected females (Kittayapong *et al.*, 2002c).

Use of the gene *wsp* sequences to characterize *W. pipientis* strains in *St. albopicta* from 12 locations (in Hawaii; East and South-east Asia; Madagascar; North and South America) showed all to be infected with both *w*AlbA (supergroup A) and *w*AlbB (supergroup B). Sequenced regions of *w*AlbA *wsp* genes from all locations were found to be identical, and the same result was obtained with *w*AlbB *wsp* genes (Armbruster *et al.*, 2003). A pattern of low mitochondrial DNA variability but average nuclear gene diversity was found within and among populations of *St. albopicta* (Kambhampati and Rai, 1991; Birungi and Munstermann, 2002). That led Armbruster *et al.* (2003) to surmise that a cytoplasmic 'sweep' caused by the superinfection of *St. albopicta* populations had homogenized the host mtDNA without affecting the diversity of their chromosomal genes.

Table 46.20 Results of crossing experiments between wild-caught males and laboratory-reared female of *Stegomyia albopicta*. The wild-caught males were from two populations (CH and KL) and were superinfected with strains of *Wolbachia pipientis* of supergroups A and B. The laboratory-reared females were of three strains: (i) UJU, uninfected; (ii) KOH, singly infected with *W. pipientis* of supergroup A (strain *w*AlbA); and (iii) KLPP, superinfected with *W. pipientis* of supergroup A (strain *w*AlbA) and supergroup B (strain *w*AlbB). (From the data of Kittayapong *et al.*, 2002c.)

Crosses	n	Mean egg hatch (%)
CH ♂ A B × UJU ♀ – –	40	0.30
CH ♂ A B × KOH ♀ A –	40	0.19
CH ♂ A B × KLPP ♀ A B	80	88.6
KL ♂ A B × UJU ♀ – –	40	0.4
KL ♂ A B × KOH ♀ A –	40	0.38
KL ♂ A B × KLPP ♀ A B	80	88.9

CH, Chachoengsao, eastern Thailand; KL, Kamchanaburi, western Thailand; *n*, number of crosses; A, supergroup A strain (*w*AlbA); B, supergroup B strain (*w*AlbB). For each cross, 35–40 wild-caught males from each location were crossed with laboratory-reared females (either uninfected – UJU; or infected with *w*AlbA – KOH) and then with laboratory-reared, superinfected females (*w*AlbA and *w*AlbB – KLPP).

It has generally been assumed that where mosquito populations carry single *W. pipientis* infections, i.e. are not superinfected, the symbiont populations are clonal and have limited capability for evolutionary change. Should a mutation result in a new *Wolbachia* variant, the progeny of the host become infected with the novel variant only. Further, cytoplasmic incompatibility selects against the loss of *W. pipientis* infections: there is a heavy cost to females that become aposymbiotic, because now their oocytes are incompatible with sperm from the males in the population that have retained infections. However, it has been proposed that a novel *W. pipientis* variant (B) might coexist with an existing infection type (A) as a superinfection (A + B) within a host individual. The number of cases of superinfection that have been reported from arthropod hosts is consistent with that possibility (Dobson, 2004).

(g) *Effects of male age on cytoplasmic incompatibility*

Two early studies of the effects of male age on the extent of cytoplasmic incompatibility were undertaken with crosses between two partially incompatible strains of *Cx. quinquefasciatus*, named Paris and Delhi. Crosses between Paris females and virgin Delhi males aged 1–2 days produced no viable larvae, but the number of larvae per egg raft increased progressively from 6.5 per egg raft when males aged 6–7 days mated for the second time, to 44.9 per egg raft when males aged 16–17 days mated for the fourth time. The effects of male age appeared to be less when all males were virgin, irrespective of age (Singh *et al.*, 1976; Krishnamurthy *et al.*, 1977).

No effect of male age on cytoplasmic incompatibility was found with strains of *Cx. pipiens* collected in California (Rasgon and Scott, 2003).

Similarly, Duron *et al.* (2007b) found no effect of male age in crosses between incompatible strains of *Cx. pipiens s.l.* By delaying oviposition, the age of sperm at karyogamy could be increased from 11 to 23 days, but such an increase did not affect the extent of cytoplasmic incompatibility. The density of wolbachiae in whole testes was strain dependent, and increased with age; for example, in two strains it increased by 3.1–11.5-fold between 2 and 30 days post-eclosion. No changes in cytoplasmic incompatibility accompanied the increases. The investigators concluded that the 'symbiont dosage' model for cytoplasmic incompatibility in *Drosophila* does not hold for *Culex pipiens*.

With *St. albopicta* crosses, the age of singly infected male parents could, apparently, affect the extent of incompatibility. When virgin, singly infected males (strain KOH) of different ages mated with uninfected females (UJU), the extent of cytoplasmic incompatibility was high in crosses involving males aged 2–5 days, but diminished with 10-day-old males (18.3% egg hatch), and diminished further with 60-day-old males (43.6% egg hatch). In contrast, when virgin, superinfected males (KLPP) of seven age classes (from 2–5 days to 60 days old) were mated with young females (1–3 days old) that were either uninfected (UJU) or singly infected (KOH), a high degree of cytoplasmic incompatibility was observed with males of all ages (Kittayapong *et al.*, 2002c).

46.9.7 Dynamics of cytoplasmic incompatibility

In any mixed population of infected and uninfected flies, cytoplasmic incompatibility gives females infected with *W. pipientis* a reproductive advantage over uninfected females. Consider a mixed *Drosophila* population. Infected males deliver sperm that have been modified by *W. pipientis* during spermatogenesis. Such sperm are unable to complete fertilization if they penetrate oocytes of uninfected females, but if they enter infected oocytes a 'rescue' function in the ooplasm allows fertilization to proceed normally. In contrast, oocytes laid by infected females can be fertilized by sperm from either infected or uninfected males (Figure 46.21A,B). Therefore, in mixed populations of infected and uninfected individuals, infected females will produce the greater number of offspring, and in each successive generation the percentage of infected individuals will rise, driven partly by the increase in number of infected males. Eventually, if maternal transmission is ideal, all individuals in the population will carry *W. pipientis*. Observations on *Drosophila simulans* (below) have shown how cytoplasmic incompatibility enables *W. pipientis* to invade natural populations.

Where mosquito populations are infected with wolbachiae of two different cytoplasmotypes that express unidirectional incompatibility, as might be at a boundary between populations, females infected with one strain of *W. pipientis* may have a reproductive advantage over the other. The dynamics of unidirectional cytoplasmic incompatibility are thought to be similar to those of *D. simulans* populations, such that in a mixed population one of the *Wolbachia* cytoplasmotypes will replace the other (Sinkins and O'Neill, 2000).

A superinfected line of *D. simulans* was produced by microinjection with two strains of *Wolbachia* that exhibited bidirectional incompatibility. Crosses between singly infected females and doubly infected males were sterile; therefore the females were unable to rescue the cytoplasmic incompatibility produced by the *Wolbachia* strain that they lacked. Crosses between doubly infected females and singly infected males were fertile irrespective of paternal infection type; therefore the females carried the strain for cytoplasmic incompatibility rescue in both cases. Thus superinfection with two strains of *Wolbachia* that are mutually incompatible results in unidirectional cytoplasmic incompatibility in crosses with hosts containing only one of the strains (Sinkins and O'Neill, 2000).

(a) Wild populations of Drosophila simulans

Knowledge of the dynamics of cytoplasmic incompatibility in wild populations of *Drosophila simulans* underpins much of our understanding of that phenomenon in mosquitoes. The very large genus of *Drosophila* comprises many subgenera,

within which species may be classified into groups and subgroups. *D. melanogaster* is the type species of the subgenus *Sophophora*, and gives its name to a group of eight species, the Melanogaster Group. Of those eight, four closely related species form a monophyletic group which is termed the Melanogaster Subgroup by some authors and the Melanogaster Complex by others who treat them as sibling species; these four are *D. melanogaster*, *D. simulans*, *D. mauritiana* and *Drosophila sechellia*. The first two are cosmopolitan in distribution, the last two are island species. All four species are hosts to *W. pipientis* (Table 46.17). Three of the species, *D. simulans*, *D. mauritania* and *D. sechellia* are chromosomally homosequential and possess very similar mitochondrial sequences, but exhibit complex phylogenetic relationships (Solignac *et al.*, 1986).

In the Hawaiian Islands, *D. simulans* were infected with *W. pipientis* of strain *w*Haw, but in southern California they were infected with *w*Ri (both of supergroup A), and these widely separated cytoplasmotypes were bidirectionally incompatible (O'Neill and Karr, 1990). The rate at which *W. pipientis* can spread through natural populations of a host was first measured in populations of *D. simulans*, and the findings were used to develop models of the dynamics of cytoplasmic incompatibility.

During the early 1980s, populations of *D. simulans* in southern California were naturally infected with *W. pipientis* (strain *w*Ri), while in central and northern California the populations were uninfected. The Californian fly populations exhibited unidirectional incompatibility: uninfected females that mated with infected males laid sterile eggs, whereas infected females produced infected progeny whether they had mated with infected or uninfected males. Sampling of *D. simulans* populations in 1984, 1990 and 1992 revealed that the *W. pipientis*-infected form had spread northwards at a rate of more than 100 km per year, and that in certain populations in which infected flies had been rare almost all flies were infected within 3 years. The dispersal of *W. pipientis* was a result of the unidirectional incompatibility. The greatest difference in fitness between infected and uninfected females lay in fecundity: in laboratory colonies, infected females were 10–20% less fecund than uninfected females, but in nature the extent of fecundity loss was less. Infection incurs a cost, and field-based estimates of variables predicted an equilibrium infection frequency of $p \approx 0.94$, which was consistent with findings from several locations (Hoffmann *et al.*, 1990; Turelli and Hoffmann, 1991, 1995).

The mitochondrial DNA of *D. simulans* occurs as two alleles, yielding haplotypes termed A and B. Sampling wild populations revealed an association between the type of mtDNA present in populations and their infection or non-infection with *W. pipientis*. In southern California, where fly populations were infected, all flies carried type B mtDNA; in northern California, where no flies were infected, all carried type A mtDNA. In the Tehachapi Mountains that separate southern from central California, the fly populations were polymorphic for mtDNA type. There, all infected flies carried type B mtDNA, while the uninfected flies carried both types. During the period 1988–1991, type A mtDNA dispersed northwards at the same rate as *W. pipientis* (Turelli *et al.*, 1992).

To test the theoretical prediction that vertical transmission, as distinct from horizontal transmission, tends to promote mutualism, studies of the Californian populations of *D. simulans* infected with *W. pipientis* (strain *w*Ri) were resumed in 2002. Tests performed in the laboratory showed that in less than 20 years, during which the flies had passed through about 200 generations, the fecundity of infected females had changed from 10–20% lower than that of uninfected females to an average of 10% greater than that of uninfected females. In both the original and the 2002 investigations, quantitatively similar but much smaller differences of relative fecundity were measured in nature. It was acknowledged that fecundity is a variable trait, differing from generation to generation. Measurements during 2002 of infection frequency in four populations gave a mean infection frequency (p) of $p \approx 0.93$, not significantly different from that determined in 1992–1993 (Weeks *et al.*, 2007).

Mathematical treatments of the dynamics of

unidirectional incompatibility in culicine mosquitoes developed by Caspari and Watson (1959) and Fine (1978) were later modified to take into account the findings from California. A basic model of the spread of *W. pipientis* in populations of *D. simulans* included the following variables: (i) H, the relative hatch rates from incompatible and compatible crosses ($H = 0$ if cytoplasmic incompatibility is 100%); (ii) F, the relative fecundity of infected and uninfected females ($F = 1$ if there is no effect on fecundity); and (iii) μ, the proportion of the offspring of infected females that were uninfected ($\mu = 0$ if transmission is 100%). The variable considered most likely to be affected by infection with *W. pipientis* was fecundity. The symbol p signified the prevalence rate of infection of adults with *W. pipientis* at time t. With the assumptions that matings were random, that the generations were discrete, and that in infected females $F < 1$, the prevalence rate of infection at generation $t + 1$ was expressed as

$$p_{t+1} = \frac{p_t(1-\mu)F}{1 - s_f p_t - s_h p_t(1-p_t) - \mu s_h p_t^2 F} \qquad (46.2)$$

where $1 - s_f = F$, and $1 - s_h = H$.

If an infection is to sweep through an isolated population, then the prevalence rate must first exceed an unstable equilibrium threshold value determined by the variables F, H and μ (Hoffmann and Turelli, 1997; Turelli and Hoffmann, 1999; Sinkins, 2004).

Populations of *D. simulans* in the Seychelles and New Caledonia were found to be superinfected with two *W. pipientis* strains (*w*Ha and *w*No) which were mutually incompatible (Merçot *et al.*, 1995). The possible competition between a superinfected and a singly infected population of *D. simulans* was tested by first developing a superinfected line from microinjected embryos, followed by a single introduction of superinfected flies into cage populations that were singly infected with either *w*Ri or *w*Ha (two bidirectionally incompatible strains). That led to 90% replacement of the singly infected flies within 12 generations (Sinkins *et al.*, 1995; Sinkins and O'Neill, 2000, review).

(b) Populations of Cx. pipiens *and* Cx. quinquefasciatus

In central California, a broad hybrid zone exists between populations of *Cx. pipiens* in the north and populations of *Cx. quinquefasciatus* in the south. Within the hybrid zone, both gene flow and hybridization occur (Urbanelli *et al.*, 1997; Cornel *et al.*, 2003). The first study of the dynamics of cytoplasmic incompatibility in natural mosquito populations was undertaken in California, during 1999 and 2000, when data were obtained from populations of *Cx. pipiens* and *Cx. quinquefasciatus* at 14 locations within the latitudinal range 33° 43′ N to 40° 27′ N. *W. pipientis* was present at all locations, and the mosquito populations were infected with only the strain *w*Pip, so just one crossing type (cytoplasmotype) was present. To analyse the data, Rasgon and Scott (2003) used a rewritten form of Eqn. 46.2, such that

$$p_{t+1} = \frac{p_t(1-\mu)F}{1 - p_t(1-F) - (1-H)(1-p_t)p_t - \mu F p_t^2(1-H)} \qquad (46.3)$$

The prevalence rate of *W. pipientis* infections at most of the 14 locations was 100%, but at three it was lower. The mean prevalence rates in 1999 and 2000 were 0.996 and 0.988, respectively, so the symbiont was at or close to fixation throughout the area. Among wild-caught gravid females induced to oviposit in the laboratory, the mean rate of *W. pipientis* transmission was 0.986 (range 0.893–1.0/♀). Therefore, the proportion of uninfected offspring of infected females had the mean value $\mu = 0.014$. In both laboratory and field experiments, no evidence was found of *W. pipientis* affecting fecundity. So, in both prevalence rate and fecundity, the *Culex* populations differed dramatically from those of *D. simulans*.

Given estimated values for cytoplasmic incompatibility (calculated from a relative hatch rate of $H = 0$ and a fecundity of $F = 1$), Eqn 46.2 could be simplified to

$$p_{t+1} = \frac{p_t(1-\mu)}{1-p_t+p\frac{2}{t}(1-\mu)} \quad (46.4)$$

Equation (46.3) predicts three equilibrium points for the prevalence rate of *W. pipientis* in the Californian populations, of which one, $p = 0$, is trivial and can be ignored. A second, $p = 1$, is a stable equilibrium point, and suggests that, after successful invasion of a population, *W. pipientis* would eventually reach fixation. The third predicted equilibrium point, i.e.

$$p' = \frac{\mu}{1-\mu} \quad (46.5)$$

is unstable. Equation 46.5 describes the threshold prevalence rate (p') that must initially be exceeded if *W. pipientis* is to invade a host population successfully. If the prevalence rate of infection exceeds this unstable equilibrium point, *W. pipientis* should spread and reach the stable equilibrium, i.e. 1 or fixation. Given the mean value of $\mu = 0.014$, and a prevalence rate $p > 0.0142$, *W. pipientis* should spread; and, where $p' = 0.05$, it should reach fixation in about 30 generations (Rasgon and Scott, 2003). The investigators commented that the simplicity of the population dynamics just described limits their usefulness for testing hypotheses concerning introductory thresholds and equilibrium levels. Had the variables had less 'ideal' values, the data that they had obtained would be more useful.

Where neighbouring, singly infected populations that are bidirectionally incompatible overlap, producing a mixed population, neither may have a mating advantage. However, if the prevalence rate of one crossing type is greater than that of the other, it will displace the other.

(c) *Populations of* Stegomyia albopicta

Most natural populations of *St. albopicta* are superinfected with strains *w*AlbA and *w*AlbB; only rarely is a population singly infected. Where two neighbouring populations are both superinfected, neither has a mating advantage. Where a superinfected population invades a population infected with only *w*AlbA (as is thought to have occurred on certain islands), the superinfected population has a mating advantage because females infected only with *w*AlbA are incompatible with superinfected males. Presumably their oocytes lack a rescue factor compatible with *w*AlbB-modified sperm (Sinkins *et al.*, 1995; Dobson *et al.*, 2001). Because superinfected females are fully compatible with *w*AlbA males, the cytoplasmic incompatibility is unidirectional and the superinfected population can spread through a population singly infected with *W. pipientis* *w*AlbA (Dutton and Sinkins, 2004). This had been observed in laboratory populations of *D. simulans* when, over a number of generations, superinfected flies progressively replaced singly infected flies (Section 46.9.7.a).

Studies with wild-caught and laboratory-reared adult *St. albopicta* showed a very high penetrance of cytoplasmic incompatibility and an absence of strain segregation, even when reared under stressful conditions. Laboratory studies with superinfected and aposymbiotic strains of *St. albopicta* showed an increase in fecundity in superinfected relative to aposymbiotic females (Section 46.9.4.e).

Noting that infection with *W. pipientis* does not diminish the fecundity of *St. albopicta*, Dobson *et al.* (2002a) modified Eqn 46.2 and wrote a basic equation for the spread of *W. pipientis* in *St. albopicta* populations in the form

$$p_{t+1} = \frac{p_t(1-\mu)}{\alpha(1-p_t)\left[p_t H+(1-p_t)\right]+p_t^2\mu\,(H-1)+p_t} \quad (46.6)$$

According to Turelli-Hoffmann population dynamics, only a very low threshold prevalence rate need be exceeded for spread of the symbiont to begin (Dobson *et al.*, 2002a; Kittayapong *et al.*, 2000, 2002a; Dutton and Sinkins, 2004).

The species of *Culex* and *Stegomyia* that in wild populations show *W. pipientis* prevalence rates of about 99%, and exhibit 100% sterility in incompatible crosses, have much more favourable dynamics of spread than that shown in the

Drosophila simulans model, and also give greater consistency between field and laboratory observations (Sinkins, 2004).

46.9.8 Bacteriophages of *Wolbachia*

(a) Characteristics and classification of bacteriophages

Bacteriophages, or phages, are tailed viruses of the Order Caudovirales that infect bacteria. The order comprises three families – the Myoviridae, Siphoviridae and Podoviridae. Over 4500 bacteriophages have been described, but for only a few is sufficient known of their virion structure, genome organization and replication for them to be assigned to a family and genus (Fauquet *et al.*, 2005). The bacteriophages of *W. pipientis* were not listed by Fauquet *et al.* (2005).

Virion. The virus particle, which has no envelope, consists of two parts – head and tail. The head is a protein shell and contains the genome; the tail is a protein tube. During infection of a susceptible cell, a phage attaches by the distal end of its tail to receptors on the cell surface, when DNA and specific proteins are injected through the tail.

Genome. This has the form of one molecule of linear dsDNA in a tightly packed coil without bound proteins; it consists of 18–500 kbp and encodes 27 to >600 genes.

Replication. Phages are either 'lytic' or 'temperate'. After entering a cell, a lytic virus either remains linear or circularizes. DNA replication results in the formation of many new virus particles, and leads to lysis of the host cell and release of the particles. This is a 'lytic infection'. After a temperate phage has entered a cell it follows one or other of two courses. (i) It may enter upon a 'lytic infection' and replicate as circular or linear extrachromosomal elements or 'plasmids'. This course leads to cell lysis. (ii) It may enter upon a 'lysogenic infection' and integrate into the host genome. Integrated viral genomes are called prophages, and the genes that they express, which are called 'lysogenic conversion genes', can be considered additional bacterial genes. Usually, their products alter the characteristics of the bacterial host. A lysogenic infection can change to a lytic infection, either spontaneously or by induction, as upon exposure to UV radiation. Then a cycle of replication begins, and after lysis of the host cell the new virions escape (Fauquet *et al.*, 2005).

Hosts. Bacteriophages have been found in over 140 genera of bacteria. They are hardly known from endosymbiotic (intracellular) bacteria, but have been described from two unrelated bacterial endosymbionts that infect invertebrate hosts. One of these is *Wolbachia*, an endosymbiont present in a wide range of insect orders, certain other arthropods and some filarial nematodes (Section 46.9.3).

(b) Phages of Wolbachia

Phages harboured by *Wolbachia pipientis* are named WO phage (Section 46.9.3.b), and occur both as free particles in the cytoplasm (lytic) and as prophage integrated into the chromosome. In an early study, electron microscopy revealed free virus particles in the ovaries of *Cx. pipiens*, specifically within some oocytes and nurse cells in swollen and lysing *W. pipientis*. The particles consisted of a head and an occasionally seen tail, characteristic of bacteriophages. The head was ~33.5 nm wide; the tail was 13.5–24.0 nm long and 13.5–19.0 nm wide. The DNA was tightly packed and had the form of a toroid (Wright *et al.*, 1978). Homogenization and fractionation of *Cx. quinquefasciatus* ovaries yielded phage-like particles, which had an icosahedral head 40–50 nm wide and a tail up to 15 nm long. Such particles were absent from the ovaries of an aposymbiotic strain (Sanogo *et al.*, 2006).

From analyses of sequence similarities between genes of phages from bacteria of seven genera (not including *Wolbachia*), Hendrix *et al.* (1999) concluded that the great majority of dsDNA tailed phages have common ancestry and that they undergo exchange of functional genetic elements drawn from a large pool. Comparisons of nucleotide sequences in the genome of phage WO (from *W. pipientis*) with sequences from other viruses revealed some interesting similarities, notably between the open reading frames designated *gp15*, *gp17* and

gp18, and the genes B and D of phage λ (presumably one of the 'λ-like viruses', family Siphoviridae), and between *gp24–26* and phage P2 (presumably one of the 'P2-like viruses', family Myoviridae) (Masui *et al.*, 2000).

Investigations into *Wolbachia* (WO) phages found no congruence between the phylogenies of WO phages and the *Wolbachia* strains that they infected, or between the phage phylogenies and those of the bacteria's insect hosts. This indicated that WO phages can be transferred horizontally between different strains of *Wolbachia*. It was also taken to show that WO phages do not cause the varied effects of the different *Wolbachia* strains on their hosts, and that WO phages can transfer horizontally between different insects with or without their bacterial hosts (Gavotte *et al.*, 2007). Coupling the evidence for a high degree of recombination in the chromosomal genes of *Wolbachia* with the evidence of recombination in phage and IS elements, Baldo *et al.* (2006a) concluded that a number of *Wolbachia* strains may be chimeras.

The *orf7* fragment, which encodes a minor capsid protein (ORF7), is a component of WO phage that has been detected in *W. pipientis* infecting a number of insects (Masui *et al.*, 2000). It was present in strains of *Cx. pipiens* infected with *W. pipientis*, but absent from an aposymbiotic strain (Sanogo and Dobson, 2004). Analyses of *orf7* sequences have revealed the complexity of WO phage, for example disclosing five different phages in six strains of *W. pipientis* (Gavotte *et al.*, 2004). Sequencing cloned *orf7* amplicons revealed nine sequences that clustered into three groups, *orf7a*, *orf7b* and *orf7c*. Hybrid offspring from crosses between two *Cx. pipiens* strains (CQ and CR) displayed a pattern of *orf7* amplification products that resembled those of the mother but that were distinct from those of the father, indicating that the *orf7* loci were maternally inherited (Sanogo and Dobson, 2004).

WO phage occurs in a high percentage of arthropod species infected with *Wolbachia*. It was found in 35 of 39 strains of *Wolbachia* from arthropod hosts. The WO phages from supergroup A or B *Wolbachia* were identified as WO-A and WO-B, respectively. Little is known about the presence of WO phage in *Wolbachia* of supergroups other than A and B, but it was absent from wolbachiae infecting the filarial nematodes *D. immitis* (supergroup C) and *B. malayi* and *Litomosoides sigmodontis* (supergroup D) (Foster *et al.*, 2005; Gavotte *et al.*, 2007).

WO-B was reported from *W. pipientis* infecting *Cx. pipiens* and *Cx. quinquefasciatus* (Bordenstein and Wernegreen, 2004). Later, by PCR detection of *orf7*, the presence of WO-B in strains of *W. pipientis* symbiotic in natural mosquito populations was determined. It was detected in 'high quantity' in species of the genera *Armigeres*, *Coquillettidia*, *Culex*, *Downsiomyia*, *Ochlerotatus*, *Stegomyia*, *Mansonia* and *Tripteroides*, with strains of *W. pipientis* from both supergroups A and B being represented. Phylogenetic analysis of the data produced trees in which nine or ten clusters of lineages from culicid hosts were apparent, some of the clusters including lineages from other insect hosts (Chauvatcharin *et al.*, 2006). No prophages in the *w*Mel or *w*Pip genomes show any signs of recent introduction, suggesting that they have been present in those symbionts for a long time (Klasson *et al.*, 2009).

WO sequences present as multiple copies dispersed in the genome of *w*Pip showed a striking polymorphism. These prophage variants provided markers for searches for polymorphism in *Wolbachia*, and more than 60 variants were found in laboratory colonies and wild populations of *Cx. pipiens*. At least 49 variants were found among populations of *Cx. pipiens* in southern Europe, and up to ten were found in a single population. In contrast, only ten were found in North African and Cretan populations of *Cx. pipiens*. The WO polymorphism, which was transferred only maternally, was stable over time. In Tunis, one variant alone was found during a period of 10 years (Duron *et al.*, 2006b).

The *w*Mel strain of *W. pipientis* is unusual among intracellular symbionts in that its genome contains very large amounts of repetitive DNA and RNA corresponding to mobile genetic elements. Three prophage elements are also present in the genome.

One is a small pyocin-like element composed of nine genes. The other two were named *w*Mel WO-A and *w*Mel WO-B, based on their location in the genome (Wu *et al.*, 2004). Five copies of WO phage are present in *w*Pip, and are named WO-*w*Pip1 to WO-*w*Pip5 according to their position in the genome. All are more closely related to the WO-B than to WO-A of *w*Mel (Klasson *et al.*, 2008).

Appendix 1

Genera and higher taxa of the family Culicidae cited in this volume, with the accepted abbreviation of generic names

All names are consistent with the online Mosquito Taxonomic Inventory (http://mosquito-taxonomic-inventory.info/). The nomenclature for taxa of the tribe Aedini accords with the revised, phylogenetic classification of that tribe. For comparison with aedine names as cited in the traditional classification and nomenclature, see Appendix 2. The scientific names of all culicid species mentioned in this volume can be found in the Species Index.

ANOPHELINAE

Tribe Anophelini
Anopheles Meigen, 1818 [An.]

CULICINAE

Tribe Aedini
Aedes Meigen, 1818 [Ae.]
Aedimorphus Theobald, 1903 [Am.]
Armigeres Theobald, 1901 [Ar.]
Catageiomyia Theobald, 1903 [Cg.]
Diceromyia Theobald, 1911 [Di.]
Dobrotworskyius Reinert *et al.*, 2006 * [Db.]
Downsiomyia Vargas, 1950 [Do.]
Fredwardsius Reinert, 2000 [Fr.]
Georgecraigius Reinert *et al.*, 2006 * [Gc.]
Halaedes Belkin, 1962 [Ha.]
Haemagogus Williston, 1896 [Hg.]
Jarnellius Reinert *et al.*, 2006 * [Ja.]
Macleaya Theobald, 1903 [Mc.]
Neomelaniconion Newstead, 1907 [Ne.]
Ochlerotatus Lynch Arribálzaga, 1891 [Oc.]
Psorophora Robineau-Desvoidy, 1827 [Ps.]
Scutomyia Theobald, 1904 [Sc.]
Stegomyia Theobald, 1901 [St.]

Tribe Culicini
Culex Linnaeus, 1758 [Cx.]
Lutzia Theobald, 1903 [Lt.]

Tribe Culisetini
Culiseta Felt, 1904 [Cs.]

Tribe Mansoniini
Coquillettidia Dyar, 1905 [Cq.]
Mansonia Blanchard, 1901 [Ma.]

Tribe Sabethini
Sabethes Robineau-Desvoidy, 1827 [Sa.]
Wyeomyia Theobald, 1901 [Wy.]

Tribe Toxorhynchitini
Toxorhynchites Theobald, 1901 [Tx.]

Tribe Uranotaeniini
Uranotaenia Lynch Arribalzága, 1891 [Ur.]

*, Reinert *et al.*, 2006 is shortened from Reinert, Harbach and Kitching, 2006).
Details of only the most recent references (Vargas, 1950; Belkin, 1962; Reinert, 2000; Reinert, Harbach and Kitching, 2006) are given in the list of References at the end of this volume.

Appendix 2

Correspondence between the names of Aedine species cited in this volume according to the traditional and revised classifications

The left-hand column lists the names according to the traditional classification and nomenclature, as in the Walter Reed Biosystematics Unit's online 'Systematic Catalog of Culicidae' (http://www.mosquitocatalog.org/). The right-hand column lists the corresponding names, but without subgenera, according to the revised, phylogenetic classification, as in the online 'Mosquito Taxonomic Inventory' (http://mosquito-taxonomic-inventory.info/).

TRADITIONAL NOMENCLATURE	REVISED NOMENCLATURE
Tribe Aedini	**Tribe Aedini**
***Aedes* (*Aedes*)** *cinereus*	*Aedes cinereus*
***Aedes* (*Aedimorphus*)** *cordellieri*	*Diceromyia cordellieri*
Aedes (*Aedimorphus*) *cumminsii*	*Aedimorphus cumminsii*
Aedes (*Aedimorphus*) *fowleri*	*Aedimorphus fowleri*
Aedes (*Aedimorphus*) *ochraceus*	*Aedimorphus ochraceus*
Aedes (*Aedimorphus*) *tarsalis*	*Catageiomyia tarsalis*
Aedes (*Aedimorphus*) *vexans*	*Aedimorphus vexans*
***Aedes* (*Diceromyia*)** *flavicollis*	*Diceromyia flavicollis*
Aedes (*Diceromyia*) *furcifer*	*Diceromyia furcifer*
Aedes (*Diceromyia*) *taylori*	*Diceromyia taylori*
***Aedes* (*Finlaya*)** *alboannulatus*	*Dobrotworskyius alboannulatus*
Aedes (*Finlaya*) *leonis*	*Downsiomyia leonis*
Aedes (*Finlaya*) *litoreus*	*Downsiomyia litorea*
Aedes (*Finlaya*) *pseudoniveus*	*Downsiomyia pseudonivea*
Aedes (*Finlaya*) *rubrithorax*	*Dobrotworskyius rubrithorax*
Aedes (*Finlaya*) *subniveus*	*Downsiomyia subnivea*
***Aedes* (*Fredwardsius*)** *vittatus*	*Fredwardsius vittatus*
***Aedes* (*Halaedes*)** *australis*	*Halaedes australis*
***Aedes* (*Macleaya*)** *tremulus*	*Macleaya tremula*
***Aedes* (*Ochlerotatus*)** *annulipes*	*Ochlerotatus annulipes*
Aedes (*Ochlerotatus*) *atlanticus*	*Ochlerotatus atlanticus*
Aedes (*Ochlerotatus*) *atropalpus*	*Georgecraigius atropalpus*
Aedes (*Ochlerotatus*) *camptorhynchus*	*Ochlerotatus camptorhynchus*
Aedes (*Ochlerotatus*) *canadensis*	*Ochlerotatus canadensis*

TRADITIONAL NOMENCLATURE	REVISED NOMENCLATURE
Aedes (*Ochlerotatus*) *cantans*	*Ochlerotatus cantans*
Aedes (*Ochlerotatus*) *cantator*	*Ochlerotatus cantator*
Aedes (*Ochlerotatus*) *caspius*	*Ochlerotatus caspius*
Aedes (*Ochlerotatus*) *detritus*	*Ochlerotatus detritus*
Aedes (*Ochlerotatus*) *dorsalis*	*Ochlerotatus dorsalis*
Aedes (*Ochlerotatus*) *epactius*	*Georgecraigius epactius*
Aedes (*Ochlerotatus*) *excrucians*	*Ochlerotatus excrucians*
Aedes (*Ochlerotatus*) *fulvus*	*Ochlerotatus fulvus*
Aedes (*Ochlerotatus*) *juppi*	*Ochlerotatus juppi*
Aedes (*Ochlerotatus*) *melanimon*	*Ochlerotatus melanimon*
Aedes (*Ochlerotatus*) *scapularis*	*Ochlerotatus scapularis*
Aedes (*Ochlerotatus*) *sierrensis*	*Jarnellius sierrensis*
Aedes (*Ochlerotatus*) *sollicitans*	*Ochlerotatus sollicitans*
Aedes (*Ochlerotatus*) *sticticus*	*Ochlerotatus sticticus*
Aedes (*Ochlerotatus*) *stimulans*	*Ochlerotatus stimulans*
Aedes (*Ochlerotatus*) *taeniorhynchus*	*Ochlerotatus taeniorhynchus*
Aedes (*Ochlerotatus*) *tormentor*	*Ochlerotatus tormentor*
Aedes (*Ochlerotatus*) *vigilax*	*Ochlerotatus vigilax*
***Aedes* (*Protomacleaya*)** *hendersoni*	'*Ochlerotatus*' *s.a. hendersoni* *
Aedes (*Protomacleaya*) *triseriatus*	'*Ochlerotatus*' *s.a. triseriatus* *
Aedes (*Protomacleaya*) *zoosophus*	'*Ochlerotatus*' *s.a. zoosophus* *
***Aedes* (*Scutomyia*)** *impatibilis*	*Scutomyia impatibilis*
***Aedes* (*Stegomyia*)** *aegypti*	*Stegomyia aegypti*
Aedes (*Stegomyia*) *africanus*	*Stegomyia africana*
Aedes (*Stegomyia*) *albopictus*	*Stegomyia albopicta*
Aedes (*Stegomyia*) *bromeliae*	*Stegomyia bromeliae*
Aedes (*Stegomyia*) *calceatus*	*Stegomyia calceata*
Aedes (*Stegomyia*) *cooki*	*Stegomyia cooki*
Aedes (*Stegomyia*) *hebrideus*	*Stegomyia hebridea*
Aedes (*Stegomyia*) *katherinensi*	*Stegomyia katherinensis*
Aedes (*Stegomyia*) *lilii*	*Stegomyia lilii*
Aedes (*Stegomyia*) *luteocephalus*	*Stegomyia luteocephala*
Aedes (*Stegomyia*) *malayensis*	*Stegomyia malayensis*
Aedes (*Stegomyia*) *metallicus*	*Stegomyia metallica*
Aedes (*Stegomyia*) *neoafricanus*	*Stegomyia neoafricana*
Aedes (*Stegomyia*) *opok*	*Stegomyia opok*
Aedes (*Stegomyia*) *polynesiensis*	*Stegomyia polynesiensis*
Aedes (*Stegomyia*) *pseudoalbopictus*	*Stegomyia pseudoalbopicta*
Aedes (*Stegomyia*) *pseudoscutellaris*	*Stegomyia pseudoscutellaris*
Aedes (*Stegomyia*) *riversi*	*Stegomyia riversi*
Aedes (*Stegomyia*) *scutellaris*	*Stegomyia scutellaris*
Aedes (*Stegomyia*) *simpsoni*	*Stegomyia simpsoni*
Aedes (*Stegomyia*) *tongae*	*Stegomyia tongae*
***Armigeres* (*Armigeres*)** *subalbatus*	*Armigeres subalbatus*

TRADITIONAL NOMENCLATURE	REVISED NOMENCLATURE
Haemagogus *equinus*	*Haemagogus equinus*
Haemagogus janthinomys	*Haemagogus janthinomys*
Haemagogus leucocelaenus	*Haemagogus leucocelaenus*
Haemagogus lucifer	*Haemagogus lucifer*
Haemagogus mesodentatus	*Haemagogus mesodentatus*
Haemagogus spegazzinii	*Haemagogus spegazzinii*
Haemagogus splendens	*Haemagogus splendens*
Neomelaniconion *circumluteolus*	*Neomelaniconion circumluteolum*
Neomelaniconion jamoti	*Neomelaniconion jamoti*
Neomelaniconion mcintoshi	*Neomelaniconion mcintoshi*
Neomelaniconion palpale	*Neomelaniconion palpale*
Psorophora *columbiae*	*Psorophora columbiae*
Psorophora ferox	*Psorophora ferox*
Psorophora signipennis	*Psorophora signipennis*
Psorophora varipes	*Psorophora varipes*

*, *s.a., sensu auctorum.*

Appendix 3

Glossary of terms used in this volume relating to the epidemiology, pathology, genetics or general biology of infectious agents and their hosts

For terms that have more than one meaning, the variants appropriate to the subject matter of this volume have been selected.

Aetiology *n.* US, etiology. In epidemiology or pathology the demonstrated cause of a disease or trait; causation. Cf. Pathogenesis.

Alpha (*α*)-helix *n.* A fundamental unit of protein folding in which successive amino acids form a right-handed helical structure held together by hydrogen bonding between the amino and carboxyl components of the peptide bonds in successive loops of the helix.

Amplifying host *n.* A vertebrate species, most commonly avian or mammalian, the individuals of which when infected by a particular arbovirus* develop a viraemia of sufficient titre and duration to infect a high proportion* of blood-feeding individuals of a vector species, so increasing the proportion of infected individuals in a vector population.

Antibody *n.* A glycoprotein of the immunoglobulin* family that can combine non-covalently and in a specific manner with a corresponding antigen*.

Antigen *n.* Any agent that when introduced into an immunocompetent animal stimulates the production of a specific antibody* or antibodies that can combine with it.

Aposymbiotic *adj.* The condition of lacking symbionts in potentially symbiotic hosts.

Arboviruses *n.* Viruses that are capable of replication and multiplication in both vertebrate and arthropod hosts, and that are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by haematophagous arthropods.

beta (*β*)-configuration *n.* A stable configuration of a peptide chain within the hydrophobic core of a globular protein in which the chain is almost fully extended but slightly puckered, and is hydrogen bonded to an adjacent polypeptide chain, thus forming a beta-strand.

beta (*β*)-sheet *n.* An approximately planar array of two or more beta-strands*.

beta (*β*)-strand *n.* Two polypeptide chains in a beta-configuration*. One of the two basic elements of the secondary structure adopted by a polypeptide chain within the hydrophobic core of a globular protein. Cf. Alpha (*α*)-helix.

Binary fission *n.* Division of a prokaryotic or unicellular eukaryotic organism into two roughly equal-sized daughter cells in asexual reproduction.

Bridge vector *n.* An arthropod species that can transmit an arbovirus from a reservoir host or an amplifying host to a dead-end host. Syn., Link vector.

Case fatality rate *n.* (i) The proportion* of cases of a specified condition that are fatal within a specified time, usually expressed as a percentage. (ii) The number of deaths from a disease in a given period divided by the number of diagnosed cases of that disease in the same period × 100. Cf. Death rate.

Cladistic method *n.* A method that uses

phylogenetic hypotheses as the basis for classification of taxa, and recency of common ancestry as the sole criterion for grouping taxa. Cf. Phenetic method.

Commensalism *n.* (i) In ecology, a symbiosis* in which one species benefits from a common food supply while the other species is not disadvantaged. (ii) In parasitology, the loosest and least obligatory form of interspecific association in which two species show a minimal metabolic dependence on each other. Hence, commensal.

Cost *n.* (i) The concept of an accumulating cost to a population in terms of an increased death rate or diminished reproductive capacity. (ii) As fitness cost, a reduction in fitness*.

Dead-end host *n.* Any vertebrate species that, after infection by a given arbovirus*, does not have viraemias of sufficient intensity and duration to permit maintenance of that virus in a transmission cycle. Syn., Tangential host.

Death rate *n.* An estimate of the portion of a population that dies during a specified period. The numerator is the number of individuals dying during the period; the denominator is the number in the population, usually estimated as the mid-period population. Cf. Case fatality rate.

Definitive host *n.* A host in which a parasite lives for part of its life cycle and in which it attains sexual maturity. Cf. Intermediate host.

Effective vertical transmission rate *n.* The proportion* of the progeny from a cohort or population of females, both infected and uninfected, that is infected through vertical transmission. Cf. Vertical transmission rate.

Encephalitis *n.* Any inflammation of the brain. *pl.* Encephalitides.

Encephalomyelitis *n.* Any inflammation involving both brain and spinal cord.

Endemic *adj.* (i) In ecology, describes an organism that is native to, and restricted to, a particular geographical region. (ii) In epidemiology, describes an infectious or potentially infectious organism that occurs persistently within a defined geographical area or a specified population. (iii) In medicine, applies to diseases that are generally or constantly found among people in a particular area.

Endemicity *n.* A general measure of the prevalence of an endemic disease.

Endocytosis *n.* A process by which eukaryotic cells take up extracellular material by invagination of the plasma membrane to form vesicles that enclose the material.

Endospore. *n.* A non-motile, asexual spore* that arises internally, within a sporangium. In bacteria and fungi, this is a single cell.

Endosymbiosis *n.* Symbiosis* in which one symbiont (the endosymbiont or inhabiting symbiont) lives within the body of the other (the inhabited symbiont).

Endotoxin *n.* Any microbial toxin* that cannot easily be separated from the structure of the cell, and that remains within the organism until cell death and cell lysis. Cf. Exotoxin.

Enveloped *adj.* Of viruses, possessing an outer (bounding) bilayered, lipoprotein membrane.

Enzootic *n.* Denoting a disease that is usually low in prevalence and constantly present in an animal population within a specified area. As *adj.*, relating to or of the nature of an enzootic. Cf. Epizootic, Zoonosis.

Enzootic cycle *n.* Repeated and continuous transmission of an infectious organism between individuals of a vector population and uninfected individuals in a vertebrate population. When there is no transmission to human beings, enzootic cycles are termed 'silent' or 'inapparent'.

Enzootic vector *n.* Any vector species that is responsible for transmission of an infectious organism in an enzootic cycle. Cf. Epizootic vector.

Epidemic *n.* 1. Extensive occurrence of a transmissible disease characterized by rapid and great increases in morbidity and sometimes mortality. 2. Any sudden increase in prevalence beyond what is considered normal. Used for outbreaks of disease in human populations or among domesticated animals. As *adj.*, relating to or of the nature of an epidemic.

Epigeal *adj.* Of sites that are above the ground surface, or organisms that dwell above the ground surface. Syn., Epigean, Epigeous. *Ant.*, Hypogeal.

Epizootic *n.* Outbreak of an infectious disease in a wild animal population, characterized by an

exceptionally large number of cases relative to the number occurring earlier. Used by some authors also for outbreaks of disease in domesticated animals. As *adj.*, relating to or of the nature of an epizootic. Cf. Enzootic.

Epizootic vector *n.* Any vector species that is responsible for transmission of an infectious organism in an epizootic cycle. Cf. Enzootic vector.

Epornithic *n.* Outbreak of an infectious disease in a wild bird population.

Etiology *n. syn.* Aetiology, q.v.

Exotoxin *n.* Any toxin* that is produced by a microorganism and secreted into the surrounding medium. Cf. Endotoxin.

Experimental vector *n.* A species that has been shown to transmit an infectious agent under laboratory conditions and that does not, or is not known to, transmit that infectious agent in nature.

Extrinsic incubation period *n.* For a vector population, the mean time between ingestion of an infected blood meal and attainment of competence to transmit the pathogen or parasite by bite, measured in days. Cf. Intrinsic incubation period.

Filial generation *n.* In experimental investigations, any generation (or offspring) following the parental generation*. The first filial generation (or offspring), F_1, results from a cross of parental individuals; the second, F_2, results from selfing or crossing of F_1 individuals; and so on.

Filial infection rate *n.* The proportion* of the progeny of an infected female that is infected through vertical transmission. (The developmental stage of the progeny is not specified.)

Fitness *n.* In population genetics, a measure of the relative survival and reproductive success of a given phenotype or population subgroup. Cf. Cost.

Fomites *pl. n.* Objects or materials which are likely to carry infectious agents.

Forage ratio *n.* Of a mosquito population, the proportion* of the engorged females that had fed upon a particular host species divided by the proportion that the host species formed of the total number of available hosts in the locality under consideration.

Gallery forest *n.* A narrow strip of forest along the margins of a river in an otherwise unwooded landscape, usually savannah grasslands. Syn., Riverine forest.

Genetic differentiation *n.* The accumulation of differences in allelic frequencies between completely or partially isolated populations as a result of evolutionary forces such as selection or genetic drift.

Genetic diversity *n.* Any variation in the nucleotides, genes or whole genomes of organisms.

Gonotrophic cycle *n.* In female mosquitoes, a sequence of behavioural and physiological events, which starts with the search for a host and the taking of a blood meal, involves the maturation of a batch of oocytes, and ends with oviposition.

Gram-negative *adj.* Of prokaryotic cells with cell walls that contain relatively little peptidoglycan and that have an outer membrane composed of lipopolysaccharide, lipoprotein and other complex macromolecules; they do not stain with Gram's crystal-violet stain.

Gram-positive *adj.* Of prokaryotic cells with cell walls that consist chiefly of peptidoglycan and that lack the outer membrane of Gram-negative cells, and that stain with Gram's crystal stain.

Haemorrhagic *adj.* US, hemorrhagic. Causing, resulting from, or characterized by haemorrhage.

Haplotype *n.* (i) The set of alleles borne on one of a pair of homologous chromosomes. (ii) A cluster of alleles on a single chromosome.

Homologous *adj.* Used of characters that share an evolutionary transformation from the same ancestral character state.

Horizontal transmission *n.* Transference of an infectious agent between any two individuals not related as parent and offspring. Cf. Vertical transmission.

Host *n.* Any organism on or in which another spends part or all of its life and from which it derives nourishment or gains protection. See also Amplifying host, Dead-end host, Definitive host, Intermediate host, Reservoir host.

Hypogeal *adj.* Of sites that are below the ground surface, or organisms that dwell below the ground surface. Syn., Hypogean, Hypogeous. *Ant.*, Epigeal.

Hypothetical ancestor *n.* A reconstruction of the character states at the ingroup* node, interpreted in terms of a real ancestor of the ingroup taxa.

Icosahedron *n.* A solid rectilinear body with twenty faces. The faces of a 'regular icosahedron' are identical equilateral triangles. *adj.* Icosahedral.

Immunoglobulin *n.* A member of a group of proteins occurring in higher animals, and produced by lymphocytes, virtually all of which possess specific antibody* activity. *Abb.*, Ig

Inapparent infection *n.* The presence of infection in a host without recognizable clinical signs or symptoms.

Incidence *n.* The number of new cases of a disease or infection arising in a defined population during a stated period. (Sometimes confused with incidence rate*.)

Incidence rate *n.* (i) The rate at which new events occur in a population. (ii) In public health practice, the number of new events in a specified period divided by the number of persons exposed to risk during that period × 10^n; i.e. the rate per 10^n human years.

Infection *n.* The entry and development or multiplication of an infectious agent within the body of an animal. The result may be inapparent* or manifest.

Infectious *adj.* The state of pathogens or parasites that are competent and available for transmission.

Infectious agent *n.* Any virus or infectious organism.

Infective *adj.* The state of a host in which infectious agents* are available for transmission.

Infrapopulation *n.* All individuals of a species of parasite present in an individual host.

Ingroup *n.* The group under investigation in a cladistic analysis in order to resolve the relationships of its members. Cf. Outgroup, Sister-group(s).

Intermediate host *n.* A host* in which a parasite lives for part of its life cycle and in which it does not become sexually mature, or in which only the asexual stages occur. *Ant.*, Definitive host*.

Intrinsic incubation period *n.* Duration of the period, in days, between infection* of an intermediate host*, as by the bite of an infective vector, and the time when the pathogens or parasites have developed to the stage at which they are infectious and can be ingested by another feeding vector. Cf. Extrinsic incubation period.

Isometric *adj.* A solid object displaying cubic symmetry.

Karyogamy *n.* Fusion of the nuclei of two gametes after cytoplasmic fusion. Cf. Syngamy.

Knockout *v.* A genetic technique by which one or more specific genes are made inoperative. *adj.* Of specific genes that have been inactivated, as knockout gene.

Latent infection *n.* The persistence of an infectious agent within a host that shows no clinical symptoms, often without its demonstrable presence in the blood, tissues or secretions of the host.

Latent period *n.* (Syn., Latency) (i) The interval of time between invasion by an infectious agent* and the first manifestations of an infection*. (Syn.: Induction period). (ii) The interval of time between invasion by an infectious agent and the host becoming infective. (See also Extrinsic incubation period, Intrinsic incubation period). (iii) In untreated malaria patients, intervals of time characterized by freedom from feelings of malaise and apparent parasite absence that occur between periods of febrile paroxysms and patent parasitaemia.

Lectins *n.* Sugar-binding proteins or glycoproteins of non-immune origin which agglutinate cells and/or precipitate glycoconjugates.

Maintenance host *n.* See Reservoir host.

Malaise *n.* In medicine, a generalized feeling of discomfort, illness or lack of well-being, sometimes accompanied by a sensation of exhaustion, that is not symptomatic of any disease or ailment but sometimes marks the onset of a disease.

Maternal infection rate *n.* The proportion* of a cohort or population of adult females that is infected at emergence, the females having infected germ cells.

Maximum likelihood estimate *n.* The infection rate, expressed as infected mosquitoes per thousand, most likely to be observed given the test results and a probabilistic model (binomial distribution). The maximum likelihood estimate is given by $MLE = 1 - (1 - Y/X)^{1/m}$, where X is number of pools, Y is number of positive pools, and m is pool size.

Meninges *n.* The three membranes enclosing the brain and spinal cord.

Meningoencephalitis *n.* Inflammation of the meninges* and of the brain.

Meningoencephalomyelitis *n.* Inflammation of the meninges* and of the brain and spinal cord.

Mesophile *n.* Used of microorganisms of which the optimum temperature for growth is between 20°C and 45°C. *adj.* Mesophilic.

Meta-analysis *n.* A statistical synthesis of data from a number of independent but similar investigations, producing quantitative findings from the pooled inputs. Meta-analysis is useful when it involves investigations that alone were inadequate to demonstrate statistically significant findings, but which in aggregate could do so.

Minimum infection rate *n.* The estimated lower limit of the true infection rate expressed as the number of infected mosquitoes per thousand tested. Thus, MIR = $Y/N \times 10^3$ where Y is the number of positive pools, and N the total number of mosquitoes tested.

Monophyletic *adj.* Derived from the same ancestral taxon. Cf. Paraphyletic, Polyphyletic.

Monophyletic group *n.* A group of taxa that contains the most recent common ancestor and all its descendants, but only its descendants. Syn., Holophyletic group.

Mortality rate *n.* See Death rate.

Mosquito infectious dose *n.* The amount of a suspension of arbovirus* that would, upon inoculation into a susceptible mosquito, lead to infection* in 50% of cases. The unit is MID_{50} ml^{-1} suspension.

Multilocus sequence typing *n.* A method of determining the extent of genomic relatedness between isolates of prokaryotes, at both intraspecies and interspecies levels, by sequence analysis of gene fragments from a minimum of five housekeeping genes.

Mutualism *n.* See Symbiosis.

Natural vector *n.* A host* species that is an effective vector* of a given infectious organism (or infectious agent*) in nature, and that has been shown to meet the criteria necessary to establish that fact.

Negative-sense *adj.* (i) In a viral genome, the nucleic acid strand with a base sequence complementary to the strand that contains the protein-coding sequence of nucleotide triplets (= minus or (–) strand). (ii) Of a virus whose genome consists of a negative-sense strand. Cf. Positive-sense.

Neurotropic *adj.* (i) Having a selective affinity for nervous tissue. (ii) Exerting its principal effect on the nervous system.

Non-stable infection *n.* An infection in which no primordial germ cells of the host are infected and in which relatively few or no gametes are infected. A female with a non-stable infection transmits the infectious agent to only a proportion* of her progeny or to none. Cf. Stable infection.

Nulliparous *adj.* Descriptive of an adult female that has not laid eggs. Cf. Parous.

Oligomer *n.* Any substance that is composed of a small number, typically two to ten, of constitutional units in repetitive covalent linkage.

Open reading frame *n.* A DNA sequence consisting of triplets that can be translated into amino acids.

Opsonin *n.* A protein that, when bound to a microorganism, increases its susceptibility to phagocytosis by linking the surface of the microorganism to a specific receptor on the surface of a phagocytic cell. *v.* Opsonized.

Orthologous *adj.* Used of homologous* genes in species that evolved from a common ancestral gene. Also used of homologous proteins or structures determined by an orthologous gene present in the most recent common ancestor. Cf. Paralogous.

Outbreak *n.* An epidemic* of only localized occurrence, e.g. in a farm, village or town.

Outgroup *n.* A taxon used in a cladistic analysis for comparative purposes, usually with respect to character polarity determination. Cf. Ingroup, Sister-group(s).

Ovarian cycle *n.* In female mosquitoes, the synchronous development of a batch of sister ovarian follicles through a sequence of steps until the oocytes they contain are fully formed. In anautogenous mosquitoes, a blood meal is necessary for initiation of the later stages.

Panmictic *adj.* Used of a randomly interbreeding population. *n.* Panmixia.

Paralogous *adj.* Used of genes that arose by gene

duplication (q.v.) and evolved in parallel within a single line of descent. Also used of homologous proteins or structures expressed by paralogous genes. Cf. Orthologous.

Paraphyletic *adj.* Used of a group of taxa that contains the most recent common ancestor plus some but not all of its descendents. Cf. Monophyletic, Polyphyletic.

Parasitaemia *n.* The presence of parasites in the blood. Sometimes used quantitatively to indicate the density of parasites in the blood.

Parasite *n.* A unicellular or multicellular eukaryote that is intimately associated with and metabolically dependent upon another living organism (the host*) for completion of its life cycle, and that is detrimental to the host to a greater or lesser degree. Cf. Pathogen.

Parental generation *n.* The original generation in an experimental study. Symbol P. Cf. Filial generation.

Parenteral *adj.* Describing the managed introduction of a chemical agent, parasite or pathogen into an animal by a pathway other than the alimentary tract.

Parous *adj.* Descriptive of an adult female that has laid eggs. Cf. Nulliparous.

Pathogen *n.* (i) Any virus or infectious prokaryote that adversely affects its hosts. (ii) A medical and veterinary term for a parasite*, especially one associated with a high degree of morbidity or mortality.

Pathogenesis *n.* (i) The process by which an infectious agent produces disease in its host. (ii) The developing pathological process. Cf. Aetiology.

Pathogenicity *n.* (i) The ability of an infectious agent* to cause disease. (ii) The deleteriousness of an infection, which depends upon the transmissibility, invasiveness and toxigenicity of the infectious agent.

Phagotrophic *adj.* Feeding by ingesting or incorporating particulate organic matter.

Pharate *adj.* Of aedine mosquitoes, used of first-instar larvae that are fully formed but remain enclosed within the chorion.

Phenetic method *n.* A method of classification of taxa based on the criteria of overall structural or biochemical similarity or difference, with all characters being equally weighted and without consideration of phylogenetic history. Cf. Cladistic method.

Phenotype *n.* The observable characteristics, structural and functional, of an organism produced by the interaction between the organism's genetic potential, or genotype, and the environment in which it exists.

Phylloplane *n.* The surface(s) of a leaf.

Plasmid *n.* A double-stranded DNA molecule, usually circular, that is present in most species of bacteria and replicates independently of the chromosome.

Polyhedron *n.* (i) Any solid body bounded by four or more plane surfaces, each of three or more sides. (ii) A polyhedral inclusion body containing one or more virions of a nucleopolyhedrovirus or cypovirus. *pl.* Polyhedra. *adj.* Polyhedral.

Polyphyletic *adj.* Used of a group of taxa that is derived from two or more distinct ancestral taxa. Cf. Monophyletic, Paraphyletic.

Polythetic taxon *n.* A taxon specified by the combination of a number of characters none of which is diagnostic alone.

Positive-sense *adj.* (i) In a viral genome, the nucleic acid strand with a base sequence containing the protein-coding sequence of nucleotide triplets (= plus strand or (+)strand). (ii) Of a virus whose genome consists of a positive-sense strand. Cf. Negative-sense.

Prevalence *n.* The number of events, e.g. cases of a disease or infection, in a given population at a designated time. (The term is sometimes used to mean prevalence rate*.) Prevalence varies as the product of incidence and duration of a disease.

Prevalence 'rate' *n.* The total number of individuals that have an attribute, e.g. an infection, at a particular time, or during a particular period, divided by the population at risk of that attribute at the specified time or midway through the specified period. (This is a proportion*, not a rate*, because the individuals identified as having the attribute are not limited to new cases.)

Prokaryotes *n.* Single-celled organisms in which the genomic DNA has the form of a single circular

molecule that is not complexed with histones and not enclosed by a nuclear membrane, and that lack membrane-bounded organelles such as mitochondria, endoplasmic reticulum and Golgi apparatus.

Proportion *n.* A type of ratio in which the numerator is included in the denominator. The ratio of a part to the whole; it may be expressed as a vulgar fraction (e.g. 1/5), as a decimal fraction (0.2), or as a percentage (20%). A proportion is a dimensionless quantity. (This is one of a number of terms used in epidemiology to describe either populations subject to infection in terms of their state at any time or of the transition of their state). Symbol, μ. Cf. Prevalence, prevalence 'rate', rate.

Proteome *n.* 1. The entire protein complement encoded by a genome. 2. The entire complement of proteins produced by a particular species.

Psychrotolerant *adj.* Used of microorganisms capable of growth at low temperatures but for which the optimum temperature for growth is above 20°C.

Putative vector *n.* A host species which, on the strength of limited information, is postulated to be a vector of a given infectious agent in nature.

Rate *n.* In epidemiology, a measure of the frequency of occurrence of a phenomenon. It may be an expression of the frequency with which an event occurs in a defined population during a specified period of time, or an expression of proportions* with a time specification.

Recombination *n.* Any process that gives rise to a new combination of genes, alleles or other nucleotide sequences.

Reservoir host *n.* A redundant term with respect to transmission of arboviruses.

Reverse transcriptase-polymerase chain reaction *n.* A two-step procedure for replicating a specific piece of RNA. The RNA is first converted to cDNA by reverse transcriptase, and is then replicated by the polymerase chain reaction.

Sahel zone *n.* In West Africa, on the southern border of the Sahara Desert, a semi-arid region which supports a very dry type of savannah* with scattered, thorny trees. *adj.* Sahelian.

Saprobe *n.* An organism that thrives in water rich in organic matter. *adj.* Saprobic. Cf. Saprotroph.

Saprotroph *n.* An organism (typically a bacterium or fungus) that feeds by secreting digestive enzymes into an organic substratum and absorbing the digestion products directly into its cells. *adj.*, saprotrophic. Cf. Saprobe.

Savannah *n.* An extensive tropical or subtropical biome (biogeographical region), its vegetation dominated by grasses with varying admixtures of tall bushes and/or trees. In Africa, three types of savannah are distinguished: humid, semi-humid and dry.

sensu auctorum Latin, meaning in the sense of authors. *Abb.*, *s.a.*

sensu lato Latin, meaning in the broad sense. In taxonomy, indicates a broad inclusiveness. *Abb.*, *s.l.*

sensu stricto Latin, meaning in the strict or narrow sense. In taxonomy, indicates a restricted inclusiveness. *Abb.*, *s.s.*

Sequela *n.* A disorder or pathological condition that results from a preceding disease. *pl.* Sequelae.

Seroconversion *n.* (i) The production in a host of specific antibodies*, that are detectable in the serum, as a result of infection* or immunization. (ii) Change of the results of a serological test from negative to positive due to the development of measurable antibodies in response to infection or immunization.

Seropositive *adj.* Possessing antibodies to a particular infectious agent as the result of a previous or ongoing clinical or subclinical infection.

Serotype *n.* Infraspecific category within a virus species, defined on the basis of possession of one or more specific antigens.

Serovar *n.* Infraspecific category within a bacterial species, defined on the basis of possession of one or more specific antigens*.

Silent *adj.* See under Enzootic cycle.

Sister-group(s) *n.* (i) Two taxa that are more closely related to each other than either is to a third taxon. (ii) The taxon that is genealogically most closely relation to the ingroup. Cf. Ingroup, Outgroup.

Spore *n.* A small, usually unicellular, propagule

from which a new individual arises without fertilization (except in the case of oospores). The spores of different taxa are seldom homologous. Some spores are heat and desiccation resistant. See also Endospore. Use of the term 'spore' for the sporocysts of coccidians and the oocysts of gregarines is inappropriate.

Stable infection *n.* An infection in which virtually all primordial germ cells of the host are infected so that all or virtually all gametes derived from them are infected. A female with a stable infection transmits the infectious agent to all her progeny. Cf. Non-stable infection.

Superinfection *n.* In an animal infected with a particular organism, a fresh infection with a genetically distinct form of the same organism.

Sylvan *adj.* Associated with woods or forests.

Sylvatic *adj.* Denoting infections in animals that dwell in woods or forests.

Symbiosis *n.* The living together in close association of two different organisms. If both organisms benefit, the symbiosis is mutualistic. If only one of the organisms benefits, the symbiosis is parasitic. Hence, *n.* Symbiont, Symbiote; *adj.* Symbiotic.

Sympatric *adj.* Used of populations, species or other taxa that occur together in the same geographical area. The populations may occupy the same habitat or different habitats within the geographical area. Cf. Syntopic.

Synanthropic *adj.* Of a species or strain adapted to living close to human habitations.

Synaptonemal complex *n.* A ladder-like structure, visible in electron micrographs of nuclei, which appears briefly during meiosis, keeping homologous* chromosomes together and closely aligned. (See Volume 1, Section 1.3.) The presence of a synaptonemal complex in a nucleus is evidence of meiosis.

Syngamy *n.* The cellular union of male and female gametes. Cf. Karyogamy*.

Syntopic *adj.* Used of species or phenotypically distinct populations that occupy the same habitat and are observable in close proximity. Cf. Sympatric.

Systemic *adj.* Of infections affecting the body as a whole.

Tangential host *n.* See Dead-end host.

Toroid *n.* A body having the shape of a torus, i.e. a ring shaped like a doughnut.

Toxin *n.* Any of a variety of substances produced by living organisms that are highly poisonous to some other organisms and distinguished from simple chemical poisons and vegetable alkaloids by their higher molecular weight and antigenicity. Cf. Endotoxin, Exotoxin.

Transcript *n.* An RNA copy of a gene.

Transhumance *n.* The seasonal movement of livestock from one grazing ground to another. *adj.*, Transhumant.

Transovarian transmission *n.* A means of vertical transmission in which infectious agents that have invaded oocytes within the ovary are transmitted to a female's progeny.

Transovum transmission *n.* A postulated means of vertical transmission in which, during ovulation, infectious agents within a gravid female invade chorionated ova (oocytes) and are transmitted to her progeny.

Trans-stadial transmission *n.* The passage of an infectious organism or infectious agent from one life-cycle stage of a host individual to a later stage.

Tropical rainforest *n.* Evergreen, wet forest present in equatorial lowlands; characterized by constant high humidity and temperature, with little seasonal fluctuation of climate except precipitation; typically with a rich variety of trees of at least 30 m height and having a dense canopy, rich in lianas and epiphytes, and with little undergrowth.

Unikaryotic *adj.* The condition of a cell containing a single nucleus.

Univoltine *adj.* Having one generation per year.

Vector *n.* An organism, usually an arthropod, that transmits a pathogen from an infected to an uninfected susceptible host. If transmission results from mere contact between vector and host, or by simple transfer from contaminated, probing mouthparts, the agent is a mechanical vector. If transmission occurs only after development or replication of the pathogen within the organism, the agent is a biological vector.

Vector competence *n.* The intrinsic permissiveness of an arthropod for the infection by and replication

and transmission of a pathogen. Vector competence is quantitatively expressed as the proportion of vectors that having fed on an infective host can subsequently transmit the pathogen to another host individual.

Vectorial capacity *n.* Of a vector species, the average number of potentially infective bites that will ultimately be delivered by all the vectors feeding upon a single host in one day.

Vertical transmission *n.* The transference of an infectious agent from a parent organism to his or her progeny. Syn: Inter-generational transmission. Cf. Horizontal transmission.

Vertical transmission rate *n.* The proportion of the progeny from a cohort or population of infected females that is infected through vertical transmission. Cf. Effective vertical transmission rate.

Viraemia *n.* The presence of a virus in the bloodstream.

Virion *n.* A structurally complete virus particle.

Virulence *n.* (i) The capacity of an infectious agent to produce either severe disease or death in a particular host. The virulence can be quantified, accordingly, as the median infective dose (ID_{50}) or the median lethal dose (LD_{50}). (ii) The degree of pathogenicity. (iii) Deleterious effects of an infectious agent upon its host, with consequences for both host fitness and the fitness of the parasite.

Virulent *adj.* Of an infectious agent that is highly infectious or extremely harmful in its effects.

Viscerotomy *n.* Incision of an organ, especially post-mortem excision of a portion of the liver.

Xenodiagnosis *n.* Method of detecting infectious agents by allowing a non-infected vector to ingest infected material and later examining the vector for evidence of the infectious agent.

Zones of emergence *n.* In Africa, ecotones from which infections of yellow fever arise. They may be forest–savannah* mosaics, or forest/savannah borders, or gallery* (riverine) forests, and are found particularly in the Guinean and southern Sudan savannah vegetational zones. Characteristically, they support concentrated populations of monkeys and vector mosquitoes.

Zoonosis *n.* A pathogen-induced disease of wild or domesticated animals that can be spread to humans. *adj.* Zoonotic.

Zoophagous *adj.* Pertaining to vector species that feed on vertebrates other than humans. Some authors use the term zoophagic.

Sources (more fully detailed in the References): *Cladistics: the Theory and Practice of Parsimony Analysis* (Kitching *et al.*, 1998); *Concise Encyclopedia of Biology* (Scott, 1996); *Concise Oxford Dictionary*, 10th edn (Pearsall, 2000); *A Dictionary of Ecology, Evolution and Systematics*, 2nd edn (Lincoln *et al.*, 1998); *A Dictionary of Epidemiology*, 4th edn (Last, 2001; edn); *Dictionary of the Fungi*, 9th edn (Kirk *et al.*, 2001); *Dorland's Illustrated Medical Dictionary* (1994); *The Encyclopedia of Ecology & Environmental Management* (Calow, 1998; ed.); *Fundamental Molecular Biology* (Allison, 2007); *Glossary of Genetics: Classical and Molecular*, 5th edn (Rieger *et al.*, 1991); *Henderson's Dictionary of Biological Terms*, 11th edn (Lawrence, 1995); *Illustrated Glossary of Protoctista* (Margulis *et al.*, 1993; eds); *International Dictionary of Medicine and Biology* (1986) (Landau, 1986); *The Penguin Dictionary of Science* (Clugston 2004); *Terminology of Malaria and Malaria Eradication* (WHO, 1963). Also listed in the References: Becnel (1994); Higgs and Beaty (2005); Margolis *et al.* (1982); Margulis *et al.* (1990); Muller (1975); Snow and Gilles (2002); Turell (1988).

Abbreviations: *Abb.*, abbreviation; *adj.*, adjective; *alt.*, alternative term; *Ant.*, antonym; Cf., compare with; *n.*, noun; *pl.*, plural; Syn., synonym; *v.*, verb.
*, Indicates a term that is defined in this glossary.

Appendix 4

Abbreviations of terms and acronyms of names used in this volume

For the acronyms of names of insect viruses that are not arboviruses see Chapter 43, Table 43.2. For the acronyms of arboviruses see Chapter 44, Table 44.1.

AFLP Amplified fragment length polymorphism analysis.
ATCC American Type Culture Collection.
AVHRR Advanced Very High Resolution Radiometer.
BHK Baby hamster kidney. As in BHK $TCID_{50}$ ml^{-1}, baby hamster kidney cell 50% tissue-culture-effective doses of virus.
bp Base pair(s). Unit of double-stranded genome.
CDC Centers for Disease Control and Prevention.
CCU Colour changing unit.
CFU Colony-forming-units. As in 3×10^8 CFU ml^{-1}.
c.i. Confidence interval.
DF Dengue fever.
DHF Dengue haemorrhagic fever.
DSS Dengue shock syndrome.
EEE Eastern equine encephalitis.
EIP Extrinsic incubation period. †
ELISA Enzyme-linked immunosorbent assay
F_1 First filial generation; the offspring from a parental cross. †
F_2 Second filial generation; the offspring from a cross within the F_1 generation. †
GFP Green fluorescent protein.
ICLD Intracerebral lethal dose. As in $10^{2.2}$ mouse $ICLD_{50}$.
ICTV International Committee on Taxonomy of Viruses.
Ig Immunoglobulin. †
ITCZ Intertropical Convergence Zone.
IUBMB International Union of Biochemistry and Molecular Biology.
kb Kilobase, i.e. 1000 nucleotide residues. Unit of measurement of single-stranded genomes.
kbp Kilobase pair; i.e. 10^6 base pairs.
KO Knockout. SKO, knockout of a single gene; DKO of two genes.
LD Lethal dose. As in LD_{50} = median lethal dose.
mbp Megabase pairs, i.e. 10^6 base pairs.
MDCK Madin-Darby canine kidney (cells).
MIC Mouse intracerebral. As in $10^{10.5}$ MIC LD_{50} ml^{-1}.
MID_{50} 50% mosquito infectious dose. As in: $10^{8.7}$ MID_{50} ml^{-1}. †
MIP Mouse intraperitoneal. As in $10^{8.2}$ MIP LD_{50} ml^{-1}.
MIR Minimum infection rate. †
MLE Maximum likelihood estimate. †
MLST Multilocus sequence typing. †
mRNA Messenger RNA.
n Total.
ORF Open reading frame. †
P In genetic experiments, the parental generation. †
PCR Polymerase chain reaction.
PFU Plaque-forming unit. The number of infectious virus particles per unit volume. As in: PFU ml^{-1}.
p.i. Post infection.

PRNT Plaque reduction neutralization test.
P_w The parental generation when it was obtained, in any life-cycle stage, from the wild.
QTL Quantitative trait locus.
RAPD Random-amplified polymorphic DNA.
RFLP Restriction fragment length polymorphism.
RT-PCR Reverse transcriptase-polymerase chain reaction. †
s.a. *sensu auctorum* (Latin), meaning in the sense of authors. †
s.l. *sensu lato* (Latin), meaning in the wide sense. †
SMIC Suckling mouse intracerebral. As in: 10^4 SMIC LD_{50} ml^{-1}.
s.s. *sensu stricto* (Latin), meaning in the strict sense or narrow sense. †
SST Sea-surface temperature.
TaqMan RT-PCR Modification of RT-PCR using TaqMan primers and probes.
$TCID_{50}$ 50% tissue-culture-infective doses of virus. As in: $TCID_{50}$ ml^{-1}.
TEM Transmission electron microscopy.

†, Term defined in the Glossary (Appendix 3).

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Species Index

This index lists individual species of mosquitoes, *Drosophila*, viruses and bacteria

Bacteria

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Subject Index

For named species of mosquitoes, *Drosophila*, viruses and bacteria see the Species Index