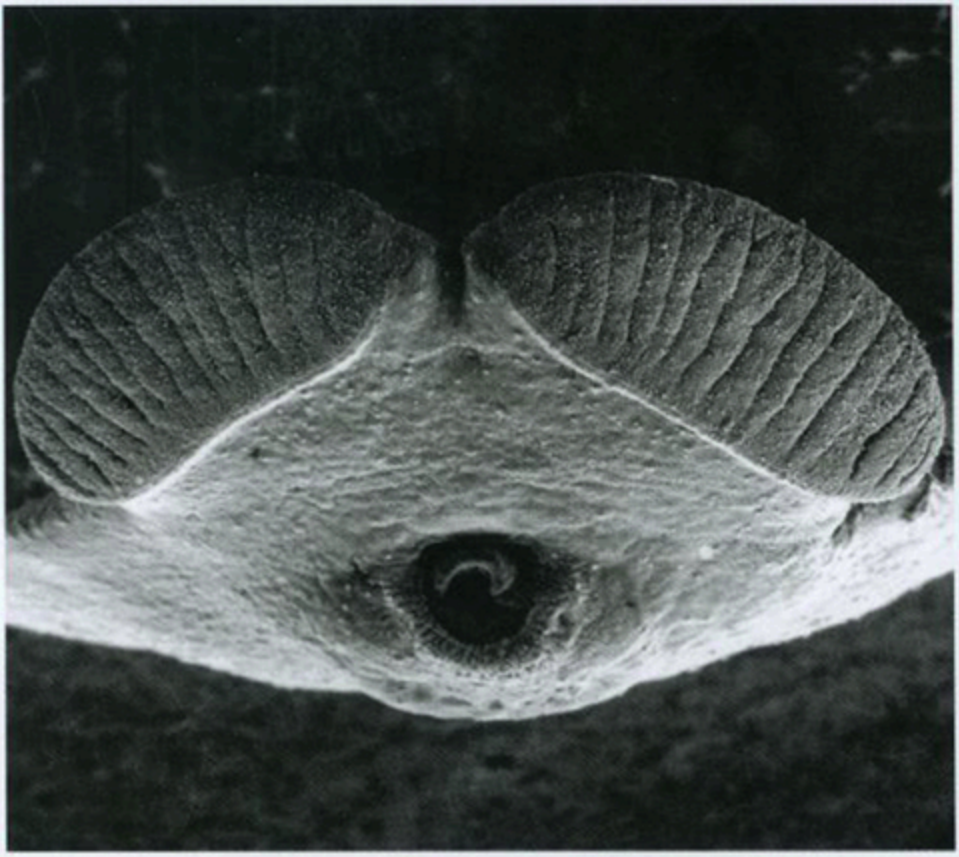
48

ADVANCES IN PARASITOLOGY



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Advances in PARASITOLOGY

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PREFACE

The volume starts with a review by Jamie Stevens (University of Exeter, UK) and colleagues of the evolutionary history of the Trypanosomatidae as revealed by current molecular studies, including DNA sequencing. The old argument between proponents of the 'invertebrate first' and 'vertebrate first' theories of the original hosts of the family seems to have been largely spurious, since it appears that the transition between monogenetic and digenetic life cycles, in whichever direction, may have occurred several times during the evolution of the family. Both monogenetic and digenetic genera seem to be closely related and, surprisingly, the genus *Trypanosoma* apparently shares with the line leading to the monogenetic parasites of invertebrates the distinction of being among the early or 'lower' trypanosomatids. Recent studies by the authors and their colleagues have indicated that *Trypanosoma* is a monophyletic genus, with *T. brucei* having evolved only in Africa while *T. cruzi* arose perhaps in a southern supercontinent ancestral to South America and Australia since – even more surprisingly – the subgenus *Schizotrypanum* appears to be linked to a little-known species which parasitizes kangaroos. *Leishmania* and *Trypanosoma* also seem to have diverged very early in the group's evolutionary history.

Vertical transmission, whereby parasites are passed from generation to generation of hosts within a host lineage, is a strategy employed by a diverse range of parasites. In this chapter Alison Dunn, Rebecca Terry and Judith Smith (University of Leeds, UK) look at transovarial transmission by microsporidia and draw on their considerable experience with the microsporidia of arthropods. It is interesting to consider the parallels between transovarial transmission of parasites with the maternal transmission of cell organelles such as mitochondria. Vertical transmission has been observed in many different microsporidian genera but the true importance of this form of transmission may have been underestimated. In contrast to horizontal transmission, which may be pathogenic due to the release of large numbers of spores, vertical transmission is rarely associated with host pathology. This chapter reviews many aspects of transovarial transmission ranging from parasite adaptations and mechanisms of transmission to the evolutionary origins of this transmission strategy within the microsporidia. It is intriguing how transovarially transmitted microsporidia may influence the sex ratios of the host

population and the authors bring together observations and stimulating ideas concerning the evolution of virulence under transovarial transmission and discuss the wider impact of this transmission strategy on host ecology and host/parasite evolution.

Parasites face numerous challenges during their often complex life cycles; one which is common to many is the need to retain their position in or on their chosen host. It is well known that each of the three major groups of parasitic Platyhelminthes possess highly characteristic and specialized organs for mechanical attachment to their hosts but relatively little attention has been given to how attachment may be enhanced by chemical means. Ian Whittington and Bronwen Cribb (University of Queensland, Australia) bring together a vast body of information concerning 'bioadhesives' secreted by Platyhelminthes. Whereas bioadhesives in many groups of invertebrates, such as marine molluscs, have been well studied this is probably the first time that such a detailed account has been given concerning the Platyhelminthes. The authors draw our attention to many fascinating questions concerned with a parasite's ability to adhere to epithelial surfaces such as fish epidermis and the lining of the vertebrate gut. For example, how is adhesion to a living surface generated so rapidly? The review considers in detail attachment by adhesives in the Turbellaria and the Monogenea and provides an insight into gland cell and possible adhesive secretions in the endoparasitic Cestoda and Digenea. It is amusing to record that this most comprehensive contribution by Ian and Bronwen, which suggests many new lines of investigation on tissue adhesion by parasites, soon became known as 'the glue review' during production.

The fourth review, by Christoph Hatz (Swiss Tropical Institute, Basel, Switzerland) deals with the use of ultrasonography in the study of the pathology of schistosomal infections. The technique has proved to be very useful in assessing the extent and development of lesions and monitoring the progress of individual chemotherapy and of community control. Its non-invasive nature, safety and the availability of portable apparatus makes it suitable for use in village clinics and in the field as well as in hospitals, although the need for trained and experienced staff to produce and interpret the ultrasonograms is a potential limitation. The use of ultrasound in studying the pathology of the five species of *Schistosoma* which infect humans is thoroughly reviewed, together with summaries of the resulting observations. The chapter concludes with a discussion of the contribution of ultrasonography to the design and monitoring of control programmes, much of which is based on the author's own experience.

As more research is carried out on the ubiquitous human roundworm infection, ascariasis, the more its medical and economic importance is being recognized. David Crompton (Glasgow University, UK) has reviewed the wealth of new information which is available on the distribution of *Ascaris* and on the morbidity and mortality caused by it. Estimates of its prevalence rise inexorably and now stand at 1400 million worldwide. The author stresses

particularly the recent findings showing that the most important effects are on children, whose weight, height and probably cognition in many countries are clearly deleteriously affected by the presence of infection.

The author has been closely involved in the formulation of control strategies (he believes that eradication is unfeasible until sanitation in many countries is improved greatly) and he authoritatively reviews measures which are proving, or could prove, effective, including their economic implications. He also deals with many fascinating aspects of the biology of *Ascaris* such as the relationship between the human and pig parasites and the possible evolution of the former, the route of migration in the human body and recent work on the immunology of infection, including allergic responses of the host.

J.R. Baker
R. Muller
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CONTENTS

The Molecular Evolution of Trypanosomatidae

J.R. Stevens, H.A. Noyes, C.J. Schofield and W. Gibson

Transovarial Transmission in the Microsporidia

A.M. Dunn, R.S. Terry and J.E. Smith

Ascaris and ascariasis

D.W.T. Crompton

Ascaris and ascariasis

D.W.T. Crompton

The Molecular Evolution of Trypanosomatidae

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ADVANCES IN PARASITOLOGY VOL 48
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ABSTRACT

In the absence of a fossil record, theories relating to the evolution of protozoa have, for most of the twentieth century, been based on morphological and life cycle data despite their known limitations. However, recent advances in molecular methodology, notably the wide availability of accurate, automated DNA sequencing, have made it possible to deduce the evolutionary relationships of extant species from their genes. This paper focuses on new findings concerning the evolution of the Trypanosomatidae, based on the ever-expanding body of molecular data now available.

Classically, the evolution of digenetic parasitism in kinetoplastids has centred around two opposing theories – invertebrate first or vertebrate first – depending on which was the original host of the monogenetic parasite. However, data supporting a close phylogenetic relationship between genera of monogenetic insect parasites and digenetic vertebrate parasites challenge the simplicity of these hypotheses and suggest that the transition may not have been a major evolutionary barrier. The implications of these observations for the evolution of parasitism within the group are discussed.

Phylogenetic analysis of a diverse selection of trypanosomatid species suggests that the genus *Trypanosoma* is monophyletic and that the human parasites, *T. brucei*, *T. cruzi* and *Leishmania* spp., have fundamentally different patterns of evolution. *T. brucei* clusters with mammalian trypanosomes of African origin, suggesting an evolutionary history confined to Africa. *T. cruzi* shows association with trypanosomes from bats, *T. rangeli*, and trypanosomes from a range of South American mammals and an Australian kangaroo. The origins of most parasites within this clade lie in South America and Australia, suggesting an ancient southern super-continent origin for *T. cruzi*, possibly in marsupials. The divergence between the *Leishmania* and *Trypanosoma* lineages is also ancient. The topology of *Leishmania* phylogenies suggests an independent transition to digenetic parasitism, a neotropical origin and an early tertiary radiation of the parasite.

1. INTRODUCTION

Parasites within the family Trypanosomatidae have either a mono- or digenetic life cycle. It seems intuitively obvious to expect the digenetic parasites to have more complex evolutionary histories than trypanosomatids with a single host, since the evolutionary pressures on two different hosts would be more varied and cumulatively greater. For most of the twentieth century, ideas on trypanosomatid evolution have had to be based on morphological and life cycle

data, despite their known limitations (reviewed by Baker, 1963 and Hoare, 1972). But the advent of molecular evolutionary methods has now made it possible to deduce the evolutionary relationships of extant species from their genes.

Since the first broad molecular study of eukaryote evolution, which included only a single representative of the genus *Trypanosoma* (Sogin *et al.*, 1986), phylogenetic analysis of kinetoplastid flagellates has become successively more focused. Initial studies concentrated on the origins of parasitism in the group (Lake *et al.*, 1988; Fernandes *et al.*, 1993) and later work on detailed analyses of evolutionary relationships among *Trypanosoma* and *Leishmania* species (Maslov *et al.*, 1996; Croan *et al.*, 1997; Lukeš *et al.*, 1997; Haag *et al.*, 1998; Stevens *et al.*, 1998, 1999a, b). In parallel, work on the evolutionary relationships of important vector groups has also developed – especially amongst Triatominae and Phlebotominae. As the level of focus has deepened, the number of species representing each genus in successive studies has increased and, significantly, there has been an associated progression of ideas concerning the evolutionary relationships between the species.

In this review we describe the contribution of the molecular evolutionary approach firstly to trypanosomatid evolution in general and then to the evolution of the genera *Trypanosoma* and *Leishmania* in detail. This is a burgeoning field and we anticipate that many more studies will be published in the near future, further expanding our understanding of trypanosomatid evolution.

2. THE EVOLUTION OF THE TRYPANOSOMATIDS

2.1. Kinetoplastids

Kinetoplastids are protozoan flagellates characterized by the kinetoplast, an organelle unique to this group which contains the mitochondrial deoxyribonucleic acid (DNA) and is located at the base of the flagellum (Vickerman, 1976). All kinetoplastids examined so far share two other unique features: compartmentalization of glycolysis within a microbody, the glycosome (Opperdoes and Borst, 1977; Opperdoes *et al.*, 1988; Michels and Hannaert, 1994), and trans-splicing of a highly conserved short ribonucleic acid (RNA) leader sequence – the spliced leader or miniexon – on to every messenger RNA (Campbell *et al.*, 1984; De Lange *et al.*, 1984; Nelson *et al.*, 1984; Muhich *et al.*, 1987; Campbell, 1992). Thus, it appears that the kinetoplastids comprise a single evolutionary lineage and the recent ribosomal RNA phylogeny presented by Wright *et al.* (1999) supports this view. Euglenids, e.g. *Euglena gracilis*, are considered to be the nearest relatives by virtue of metabolic and

structural similarities (Vickerman, 1994), but do not have either kinetoplast (Vickerman, 1994) or glycosomes (Michels and Hannaert, 1994). Molecular evolutionary analysis, based on sequence comparison of the small subunit ribosomal RNA (ssu rRNA) genes (Schnare *et al.*, 1986; Sogin *et al.*, 1986), glycolytic enzyme genes (Michels and Hannaert, 1994) and elongation factor 1α (Hashimoto *et al.*, 1995), indicates that kinetoplastids (represented by the genera *Leishmania*, *Crithidia* and *Trypanosoma*) are one of the earlier diverging eukaryote lineages after acquisition of the mitochondrion.

In this paper we adhere to the generally accepted taxonomic approach which subdivides the order Kinetoplastida Honigberg, 1963 into three families: Trypanosomatidae, Bodonidae and Cryptobiidae (Vickerman, 1976; Levine *et al.*, 1980). Trypanosomatids have a single flagellum and all genera are parasitic in vertebrates, invertebrates, ciliates or flowering plants (Table 1). Bodonids and cryptobiids typically have two heterodynamic flagella. Most bodonid species are free-living inhabitants of aqueous environments, e.g. *Bodo* spp., but within these two families there are also vertebrate ecto- and endoparasites, e.g. *Ichthyobodo* spp., *Trypanoplasma* spp., *Cryptobia* spp. and invertebrate parasites, e.g. *Cryptobia* spp. Accordingly, the most parsimonious line of reasoning suggests that the common ancestor of all the parasitic kinetoplastid genera was free living, now represented by the bodonid lineage. The finding that two free living taxa, *Dimastigella trypaniformis* and *Rhynchobodo* sp., are the earliest branching clades within the ssu rRNA phylogeny of the kinetoplastids (Wright *et al.*, 1999) is consistent with this.

Table 1 The Trypanosomatidae

	Genus	Morphology	Hosts	Vectors
Monogenetic	*Blastocrithidia*	Epimastigote, amastigote, cyst	Insects, ticks	
	Crithidia	Choanomastigote	Insects	
	Herpetomonas	Promastigote, opisthomastigote	Insects	
	Leptomonas	Promastigote, cyst	Insects, nematodes, ciliates	
	Rhynchoidomonas	Trypomastigote – no undulating membrane	Diptera	
Digenetic	*Endotrypanum*	Amastigote, promastigote, epimastigote, trypomastigote	Sloths Mammals, lizards	Sandflies Sandflies
	Leishmania	Amastigote, promastigote	Flowering plants	Hemiptera
	Phytomonas	Promastigote[1]		
	Trypanosoma	Amastigote, epimastigote, trypomastigote	Vertebrates	Arthropods, leeches

[1] Vickerman (1976) noted that amastigotes have also been reported in *Phytomonas*.

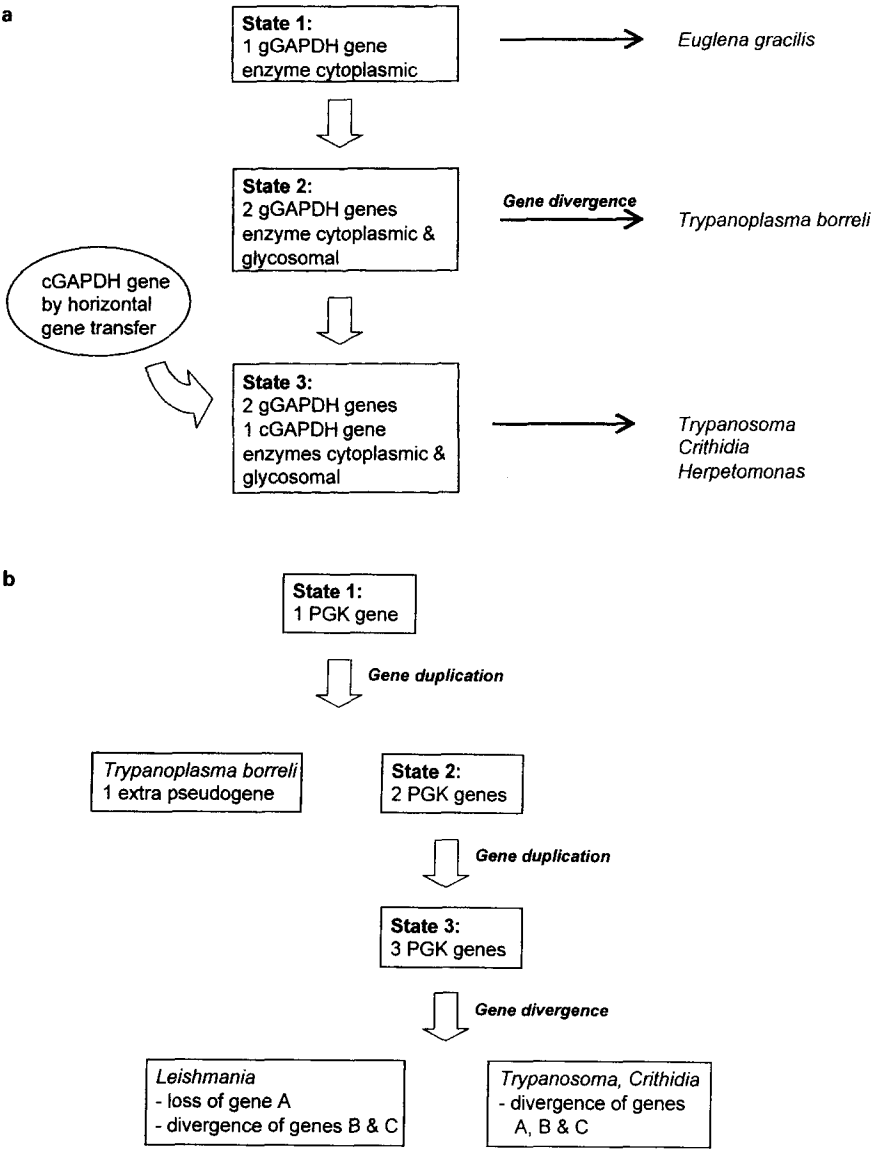

Figure 1 Glycosome evolution in Trypanosomatidae: two evolutionary scenarios. a. The pattern of gene duplication and divergence in glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes. b. The pattern of gene duplication and divergence in phosphoglycerate kinase (PGK) genes. After work by P. Michels and colleagues (see Michels and Hannaert, 1994; Wiemer *et al.*, 1995; Adjé *et al.*, 1998; Hannaert *et al.*, 1998).

2.2. Bodonids and Cryptobiids

The validity of using bodonids or cryptobiids (usually *Bodo caudatus* or *Trypanoplasma borreli*) as outgroups to root phylogenetic trees constructed from trypanosomatid gene sequences has been assumed from their taxonomic position and amply confirmed by the molecular phylogenetic studies of Fernandes *et al.* (1993), Maslov *et al.* (1994), Lukeš *et al.* (1997) and Wright *et al.* (1999) using ribosomal RNA genes, and Alvarez *et al.* (1996) and Hannaert *et al.* (1998) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes, with *Euglena gracilis* or other eukaryotes as outgroups. There has been little independent analysis of evolutionary relationships amongst the three kinetoplastid families, however, because far less information is available on the bodonids and cryptobiids than on trypanosomatids.

Perhaps the best evidence so far comes from studies on glycosome evolution by Michels and colleagues, who proposed the evolutionary scenario depicted in Figure 1 based on the pattern of gene duplication and divergence in GAPDH (Figure 1a) and phosphoglycerate kinase (PGK; Figure 1b) genes (Michels and Hannaert, 1994; Wiemer *et al.*, 1995; Adjé *et al.*, 1998; Hannaert *et al.*, 1998).

Others have begun detailed analysis of kinetoplast structure and RNA editing in bodonids (*Bodo saltans*: Blom *et al.*, 1998) and cryptobiids (*Cryptobia helicis*: Lukeš *et al.*, 1998; *Trypanoplasma borreli*: Lukeš *et al.*, 1994; Maslov and Simpson, 1994; Yasuhira and Simpson, 1996; Simpson, 1997). Ultrastructural studies have revealed that, in contrast to the tightly catenated structure in trypanosomatids, the kinetoplast DNA of bodonids (*Bodo caudatus*) and

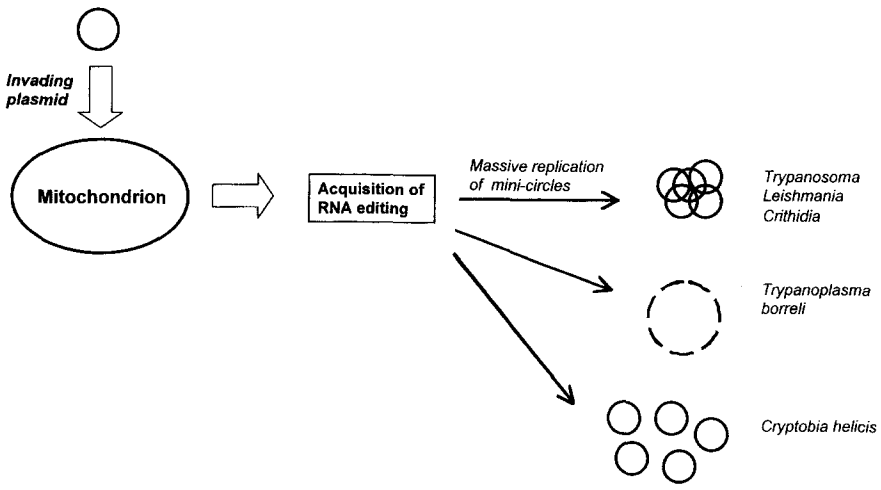

Figure 2 A model for the evolution of kinetoplast DNA, as proposed by Lukeš et al. (1998).

cryptobiids (*Cryptobia vaginalis*) is dispersed throughout the mitochondrion (Hajduk *et al.*, 1986); Vickerman (1977) coined the term pankinetoplast for this organization. Figure 2 illustrates the model proposed by Lukeš *et al.* (1998) for the evolution of kinetoplast DNA.

The evidence from both glycosome evolution and kinetoplast structure is consistent with the prevailing view that the bodonids and cryptobiids represent the ancestral forms of the order.

3. THE EVOLUTION OF PARASITISM

3.1. Evolution of Parasitism in Kinetoplastids

While it is relatively easy to construct satisfactory scenarios for the evolution of parasitism among bodonid and cryptobiid flagellates, because of the range of present day life styles this is much more challenging for the trypanosomatids, which are all parasitic. Free-living bodonids occupy a wide range of aquatic niches in terms of oxygenation and salt concentration, and ectocommensal and ectoparasitic species have colonized the outer surface of both invertebrate and vertebrate hosts. Some cryptobiids are ectoparasitic, but have also invaded various body cavities of invertebrates and vertebrates, e.g. reproductive or gastrointestinal tracts of snails, leeches or fish, and have also successfully colonized the bloodstream of fish, exploiting blood-sucking leeches for transmission. It is thus easy to envisage how the step from monogenetic to digenetic parasitism was achieved either by invasion via the skin or via the gut mucosa (Baker, 1994) and, indeed, bloodstream infections by ectoparasitic cryptobiids have been demonstrated (Woo, 1994). A third possibility is that the digenetic parasites arose from monogenetic parasites of leeches (Vickerman, 1976). Such conveniently plausible stepping stones are missing for the present day species of trypanosomatids. As with bodonids and cryptobiids, the step from free living to monogenetic parasitism seems readily achievable, the resistant cyst stage developed by free living forms being a preadaptation for transmission between hosts. However, the development of digenetic parasitic life styles is more problematic and has been a topic of controversy for most of the twentieth century. Baker (1963), Wallace (1966), Hoare (1972) and, more recently, Vickerman (1994) and Maslov and Simpson (1995) have reviewed the various arguments and hypotheses. The two opposing theories can be summarized as 'invertebrate first' or 'vertebrate first', depending on which was the original host of the monogenetic parasite. Most parasitologists tended to favour the former hypothesis (Baker, 1963, 1994; Hoare, 1972). Morphological studies suggest *Leptomonas* to be the most primitive trypanosomatid genus, with a clear progression to genus *Trypanosoma*

(Baker, 1963; Table 1), but Hoare (1972) favoured *Blastocrithidia* as the genus ancestral to trypanosomes.

New results from molecular phylogenetic studies, however, have now turned this idea on its head. There is resounding support for a close relationship between the monogenetic trypanosomatid genera *Crithidia* and *Leptomonas* and the digenetic vertebrate parasites *Leishmania* and *Endotrypanum*. This surprising result emerged from the first ribosomal RNA trees (Hernandez *et al.*, 1990; Fernandes *et al.*, 1993) and has been firmly established by subsequent ribosomal RNA and protein-coding gene phylogenies (Landweber and Gilbert, 1994; Maslov *et al.*, 1994, 1996; Alvarez *et al.*, 1996; Lukeš *et al.*, 1997; Adjé *et al.*, 1998; Haag *et al.*, 1998; Hannaert *et al.*, 1998; Stevens *et al.*, 1999a). Fernandes *et al.* (1993) concluded that digenetic parasitism had evolved four times in the Trypanosomatidae, once in each of the lineages leading to *Leishmania/Endotrypanum*, *Phytomonas*, *T. cruzi** and *T. brucei* (although later studies have shown that the genus *Trypanosoma* is monophyletic – see Section 4.2, p. 12). From their analysis of several protein-coding gene phylogenies, Alvarez *et al.* (1996) proposed that the family Trypanosomatidae has two main clades: (i) genus *Trypanosoma* and (ii) the other genera (*Leishmania*, *Phytomonas*, *Endotrypanum*, *Leptomonas*, *Herpetomonas*, *Crithidia* and *Blastocrithidia*). However, the exact branch order of *Herpetomonas* and *Phytomonas* relative to the *Crithidia/Leishmania* and *Trypanosoma* clades has yet to be determined; the confusion over monophyly/paraphyly in the genus *Trypanosoma* (see Section 4.2, p. 12) indicates the need for caution when interpreting the branching order of lineages with few representatives. In addition, species of the genus *Leptomonas*, which parasitize insects, nematodes and ciliates, may turn out to be far more diverse than presently recognized, and no representative of the genus *Rhynchoidomonas* has yet been examined; this little-known genus appears to be the most similar to trypanosomes morphologically, having trypomastigote forms lacking an undulating membrane (Vickerman, 1976). More representatives of all these genera will need to be studied before firm conclusions can be drawn about the evolution of the monogenetic parasites.

3.2. Evolution of Parasitism in the Genus *Trypanosoma*

The new molecular phylogenies are consistent with either the vertebrate or invertebrate first hypotheses, since both genus *Trypanosoma* and the branch leading to the monogenetic parasites arise from the root of the trypanosomatid tree (Figure 4; see Section 4.2, p. 12). Thus, in direct contradiction to the long-cherished belief that insect trypanosomatids are primitive, it now appears

*Throughout this review, the generic abbreviation *T.* refers to *Trypanosoma*.

that genus *Trypanosoma* is also one of the 'lower trypanosomatids'. Indeed, Landweber and Gilbert (1994) even went so far as to suggest that digenetic parasitism might be the primitive state.

The depth of the branch point between the two main trypanosomatid clades evident from a range of phylogenies based on ribosomal RNA or protein-coding genes indicates that the genus *Trypanosoma* has had a long and separate evolutionary history. Using a 'molecular clock' approach to calculate sequence similarity values (see Section 4.7.1, p. 25), Fernandes *et al.* (1993)* estimated the branch point at >340 million years before present (mybp), i.e. before vertebrates emerged on to the land and before the evolution of insects; a more recent 'clock'-based analysis by Haag *et al.* (1998) also placed the divergence of the genus *Trypanosoma* from the other trypanosomatids at well over 300 mybp. Of course, it must be borne in mind that these estimates were based on rates of evolution derived from ssu rRNA gene-based studies of the Metazoa; since there may be as much as an eight-fold difference in rates of evolution of the ssu rRNA gene within the genus *Trypanosoma* alone, it is at present impossible reliably to calibrate the trypanosomatid clock against other kingdoms with any useful degree of accuracy (see Section 4.7.2, p. 26). Nevertheless, there is no evidence against the hypothesis that trypanosomes first appeared as monogenetic parasites of aquatic vertebrates or invertebrates and subsequently adapted to digenetic transmission cycles involving aquatic vertebrates and leech vectors (Woo, 1970; Baker, 1994; Vickerman, 1994). The transition to insect vectors would have come later, following the colonization of the land by the vertebrates (~300 mybp), the appearance of the Hemiptera (~250 mybp) and the Diptera (~200 mybp) and the evolution of the blood-sucking habit among these insects (Evans, 1984). Similarly, the transition to insect parasitism is unlikely to pre-date the appearance (~300 mybp) of the Orthoptera – the first of the 10 extant insect orders that carry trypanosomatids (Wallace, 1966; Evans, 1984). It is significant that trypanosomatids do not occur in present day representatives of the earliest insect groups, the Ephemeroptera and Odonata, although both have aquatic larvae (Wallace, 1966). Wallace was also struck by the association of trypanosomatids with insect groups containing blood-sucking members, e.g. Hemiptera and Diptera, which prompted him to suggest that insect trypanosomatids had secondarily been acquired from the blood of vertebrates in this way. If the ancestral monogenetic trypanosomatids were parasites of vertebrates, it is puzzling that we do not find monogenetic trypanosomes in fish or aquatic invertebrates today, as for cryptobiids. It is possible that the digenetic life style evolved so long ago that the original monogenetic host is now extinct. On the other hand, there are present day monogenetic trypanosomes, such as *T. equiperdum*, transmitted by coitus in

*According to most recent phylogenetic analyses, the tree topology presented by Fernandes *et al.* (1993) is incorrect; nevertheless, the structural similarity values on which the estimate of divergence time is based are derived independently of tree topology, being based on aligned sequence similarity.

horses, and *T. cruzi*, transmitted by urine among opossums and congenitally or via maternal milk in humans (Miles, 1979). Indeed, in many trypanosome life cycles, there is clear evidence of the ability to pass directly into and out of the bloodstream via intact mucous membranes, e.g. in *T. cruzi*, *T. evansi*, *T. lewisi*, *T. grayi* (see Hoare, 1972). This suggests a scenario whereby trypanosomes could have evolved as monogenetic bloodstream parasites, perhaps from flagellates of the genitourinary tract of the first amphibians. The bloodstream trypanosomes would have been available to blood-sucking leech and insect vectors, while the trypanosomes in excreta or in corpses could have found their way into scavenging insects, particularly Diptera, perhaps giving rise to some of the genera of monogenetic trypanosomatids. In this scenario, trypanosomes passed secondarily into fish via infected leeches, side-stepping the problem of parallel evolution of a fish–leech digenetic cycle in both trypanosomes and cryptobiids. However, the close relationship between fish and amphibian trypanosomes evident from the molecular phylogenies (see Section 4.3, p. 15) could also be interpreted as evidence of the emergence of amphibian trypanosomes from an earlier digenetic fish–leech cycle. Arguably, the long lag between the emergence of land vertebrates (300 mybp) and that of the blood-sucking habit in insects (Psychodidae; ~200 mybp) suggests that trypanosomes would need to have adopted alternative modes of transmission during this period to have survived at all in terrestrial vertebrates.

Acceptance of a 'vertebrate first' hypothesis throws up a further problem. So far, *T. brucei* is the only trypanosomatid known to undergo some form of sexual reproduction and this occurs in the insect vector (Jenni *et al.*, 1986). The process apparently involves a meiotic division, suggesting that it is unlikely to have arisen *de novo* in *T. brucei* and is probably an ancestral feature, secondarily lost in some lineages. In which case, we should perhaps expect the insect to be the most primitive host.

3.3. Adaptation to Vertebrate Parasitism by Insect Trypanosomatids

The close phylogenetic relationship between some genera of monogenetic insect parasites and digenetic vertebrate parasites suggests that the evolutionary transition between them may not have been a major barrier and, in the case of *Leptomonas*, *Crithidia*, *Leishmania* and *Endotrypanum*, it appears to have occurred relatively recently. If so, what are the possibilities of this recurring? A number of cases of infection of animals and humans with monogenetic trypanosomatids has been reported, several of them associated with human immunodeficiency virus infections (Schnur *et al.*, 1992; Jiminez *et al.*, 1996; Pacheco *et al.*, 1998; Sousa *et al.*, 1998); similarly, the anal glands of marsupials can be infected with a range of otherwise monogenetic trypanosomatids (Deane and Jansen, 1988; Jansen *et al.*, 1988). However, this evidence needs

critical evaluation to exclude the possibilities of laboratory cross-contamination or superinfection of leishmanial lesions by monogenetic trypanosomatids – the characterization of such parasites with independent gene markers before firm conclusions can be drawn is essential. For example, one of the best documented of these parasites was isolated from a patient with acquired immune deficiency syndrome in Martinique and was described as a lower trypanosomatid on the basis of its highly distinctive isoenzyme profile and unusual morphology in the patient (Dedet *et al.*, 1995). However, sequences of the ssu rRNA gene and RNA and DNA polymerase genes indicate that this parasite was in fact a divergent member of the genus *Leishmania* (H.A. Noyes, unpublished observations). If any of these sporadic infections are proven to be due to members of the monogenetic trypanosomatid genera, it would indicate that there must be both biochemical and ecological barriers to these infections developing into established cycles. Furthermore, if novel infections with otherwise monogenetic parasites are common, it would suggest that the primary barrier is ecological rather than biochemical. The degree to which this is true has significant implications, since the changes in habitat associated with human development may create conditions that are suitable for the establishment of anthroponotic cycles of infection with parasites that have hitherto been regarded as monogenetic parasites of invertebrates.

4. GENUS *TRYPANOSOMA*

4.1. Overview

Trypanosomes are obligate parasites of all vertebrate classes and are transmitted by arthropod or leech vectors. The trypomastigote form, with a recurrent flagellum attached to the body by an undulating membrane, is found free in the bloodstream of the vertebrate host. Intracellular forms are found in some species, e.g., *T. cruzi*. Hoare (1972) subdivided the mammalian trypanosomes into salivarian and stercorarian sections, depending on their mode of development in the vector. Trypanosomes of other vertebrates have not been so well studied, indeed complete life cycles of some are unknown, and they were not therefore included in this system of nomenclature. The salivarian trypanosomes are all tsetse-transmitted and are further subdivided into four subgenera, *Trypanozoon, Duttonella, Nannomonas* and the little known *Pycnomonas*, based on their morphology and development in the vector (Hoare, 1972). From the assumed progressive adaptation of these trypanosomes to the tsetse fly (*Glossina*), Hoare (1972) proposed that the subgenus *Duttonella* was the most primitive and the subgenus *Trypanozoon* the most highly evolved. The group as a whole is characterized by antigenic variation, studied in strains of *T.*

(Trypanozoon) brucei, *T. (T.) evansi*, *T. (T.) equiperdum*, *T. (Nannomonas) congolense* and *T. (Duttonella) vivax*. These species, together with *T. (N.) simiae* and *T. (N.) godfreyi*, have similar molecular karyotypes with chromosomal DNAs ranging in size from approximately 50 to 6000 kb. An estimated 100 minichromosomes of 50 to 100 kb are present in all these species, except *T. (D.) vivax*. The minichromosomes are characterized by the presence of a highly repetitive DNA element or satellite DNA repeat of 177–500 bp in size, which is usually adenine + thymine-rich, but guanine + cytosine-rich in *T. (D.) vivax*. The only genes known to be carried by the minichromosomes are those for variant surface glycoproteins.

The stercorarian forms comprise three subgenera. Subgenus *Schizotrypanum* is well defined both by morphology and multiplication as amastigote forms within tissue cells in the mammalian host. These species are parasites of bats and a wide variety of other mammals, including humans. The rest of the stercorarian species are allotted to the other two subgenera: *Megatrypanum* – large trypanosomes that multiply as epimastigotes in the mammalian host, and *Herpetomonas* – medium-sized trypanosomes that multiply as epimastigotes or amastigotes in the mammalian host (Vickerman, 1976). It is no surprise that these two rather poorly defined subgenera now appear to be polyphyletic (see Sections 4.5. and 4.6, pp. 17 and 24).

4.2. Monophyly Versus Paraphyly

In the genus *Trypanosoma*, successive studies have included increasing numbers of species, with the effect that the phylogenetic trees have themselves progressively changed. Initial studies, indicating the genus *Trypanosoma* to be paraphyletic, have been superseded by studies with more species, which showed the genus to be monophyletic.

Early 18S ssu rRNA gene studies were summarized by Maslov and Simpson (1995) in a phylogenetic tree which included three trypanosome species, *T. brucei*, *T. cruzi* and a third species from a fish (E1–CP, Figure 3). In common with other early studies (Gomez *et al.*, 1991; Fernandes *et al.*, 1993; Landweber and Gilbert, 1994), this tree indicated the genus *Trypanosoma* to be paraphyletic. Subsequently, Maslov *et al.* (1996) increased the number of *Trypanosoma* species to seven; however, this still left *T. brucei* outside both the main trypanosome clade and the trypanosomatid clade containing *Leishmania* and *Crithidia*. The results of these early studies are summarized in Figure 3. The inclusion by Lukeš *et al.* (1997) of four more trypanosome species demonstrated for the first time that the genus *Trypanosoma* might in fact be monophyletic and the addition of more outgroup taxa considerably strengthened this conclusion. Subsequently, trees including 24 trypanosome species

(Haag *et al.*, 1998) and 47 trypanosome taxa (Stevens *et al.*, 1999a) have both supported monophyly of trypanosomes unequivocally and it seems unlikely that, at least for the ssu rRNA gene, addition of further taxa will alter this conclusion. An extended version of the tree presented by Stevens *et al.* (1999a) is given in Figure 4.

Thus the evolutionary trees have themselves evolved, spawning a progression of ideas about trypanosome evolution in the process. Early trees, which showed trypanosomes to be paraphyletic (Maslov and Simpson 1995; Maslov *et al.*, 1996), suggested that parasitism and the digenetic life cycle had arisen more than once in the trypanosome lineage. The evidence of monophyly revealed by more recent trees clearly contradicts this, but still supports the idea that parasitism and digenetic life cycles have evolved independently in several trypanosomatid lineages (see Figure 4). While the hypothesis of co-evolution of trypanosomatids and their vectors was not supported by early trees (Maslov *et al.*, 1996), later trees reveal obvious clade and vector associations; for example, trypanosomes in the aquatic clade are probably all transmitted by aquatic leeches, while *T. brucei* clade taxa (excluding *T. evansi* and *T. equiperdum* – see above) share transmission by tsetse flies (Figure 4).

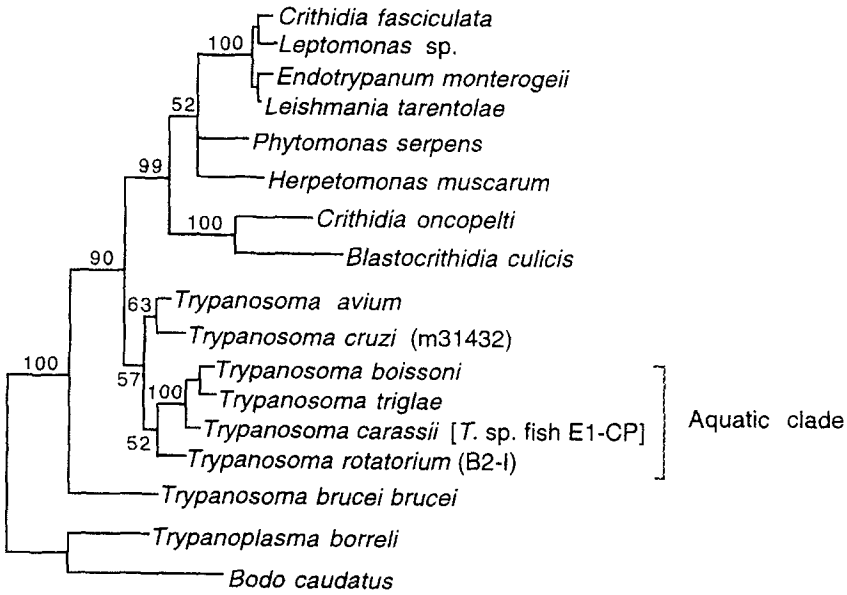

Figure 3 Bootstrapped maximum parsimony phylogenetic tree summarizing the ssu ribosomal RNA gene sequence studies by Maslov and Simpson (1995) and Maslov *et al.* (1996); the tree contains seven *Trypanosoma* species and indicates the genus *Trypanosoma* to be paraphyletic (see also Figure 4 for comparison).

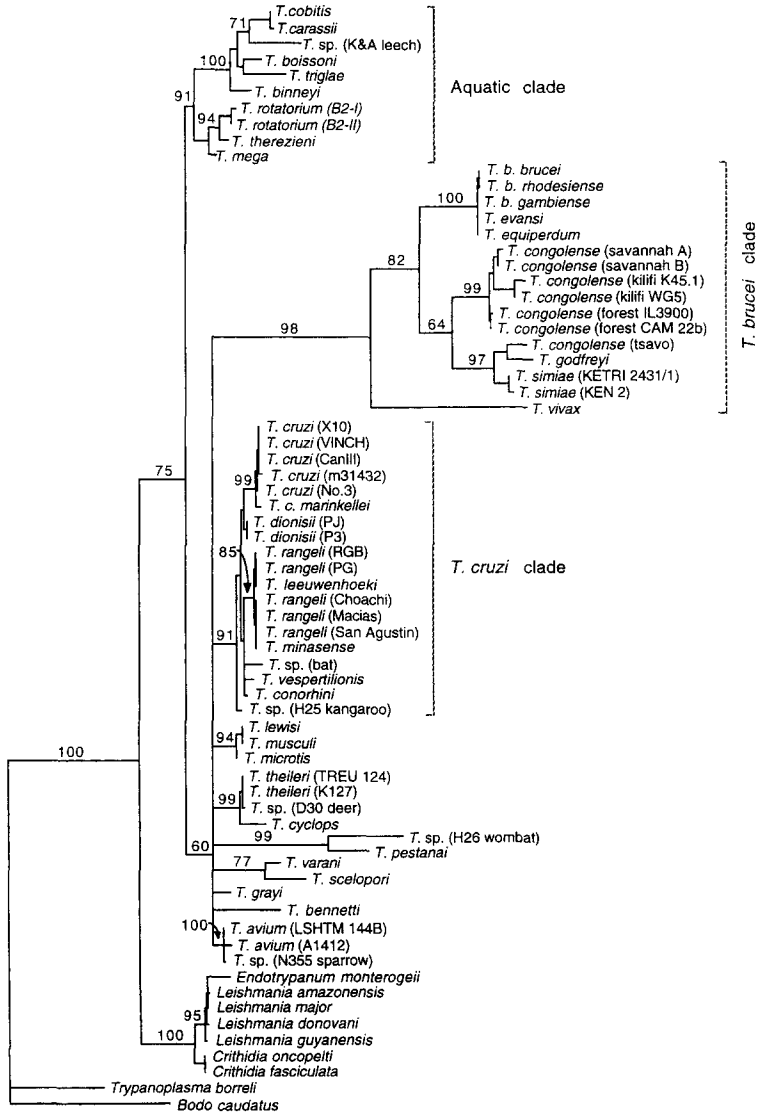

Figure 4 Phylogenetic tree based on bootstrapped maximum parsimony analysis of ssu ribosomal RNA gene sequences. The tree represents an extended analysis by Stevens *et al.* (1999a) and is based on an alignment of 1809 nucleotide positions, being one of three alignments tried (Morrison and Ellis, 1997; Stevens *et al.*, 1999a). It contains 61 *Trypanosoma* taxa and shows the genus to be monophyletic. *S*equence accession numbers are given by Haag *et al.* (1998) and Stevens *et al.* (1999a, b), except for *Trypanosoma binneyi* (AJ132351). Analysis was performed using PAUP* 4 (Swofford, 1998). See also Figure 3 for comparison – the relative branch lengths within both trees are correct, but branch lengths between trees cannot be directly compared.

In Figure 4, the ribosomal RNA data have not allowed the exact branching order of these groups to be determined and the tree shows a nine-way polytomy. Interestingly, the aquatic clade forms the first branch from the trypanosome lineage in Figure 4, providing evidence in support of host–parasite co-evolution, although the relatively low bootstrap value (60%) indicates that other hypotheses might also be considered. The polytomy and low bootstrap support suggest that the limit of the resolving power of the ssu rRNA marker over this time scale may have been reached and other markers, e.g. GAPDH, may be more informative.

In studies using 28S large subunit rRNA sequences (lsu rRNA), usually in conjunction with ssu rRNA sequences, conclusions relating to *Trypanosoma* largely agree with ssu-only studies, i.e. earlier studies using fewer taxa show paraphyly and later studies monophyly (Gomez *et al.*, 1991; Briones *et al.*, 1992; Fernandes *et al.* 1993; Landweber and Gilbert, 1994; Maslov *et al.*, 1994, 1996; Lukeš *et al.*, 1997). However, studies based on the GAPDH gene have consistently shown *Trypanosoma* to be monophyletic even when considering only two to five *Trypanosoma* species (Hannaert *et al.*, 1992, 1998; Wiemer *et al.*, 1995; Alvarez *et al.*, 1996), indicating that this gene may be a more reliable phylogenetic marker over the time scale in which the *Trypanosoma* species appear to have diverged. Similarly, studies based on 9S and 12S mitochondrial rRNA genes (Lake *et al.*, 1988), elongation factor 1α (Hashimoto *et al.*, 1995), trypanothione reductase and α-tubulin (Alvarez *et al.*, 1996) and phosphoglycerate kinase (Adjé *et al.*, 1998), and including at most five trypanosome species, also indicate the genus to be monophyletic.

4.3. The Aquatic Clade

Within the trypanosome clade, several subclades are apparent. An 'aquatic clade', comprising trypanosome species isolated from both marine and freshwater fish, amphibia and leeches, emerged even in early studies and has become progressively better defined. While little information can be gleaned from the single isolate from a fish included by Maslov and Simpson (1995), the study by Maslov *et al.* (1996), which included seven trypanosome species from fish and amphibia, clearly showed the emergence of an aquatic clade (Figure 3). In this paper we have also included in the aquatic clade ssu rRNA sequences from *T. binneyi* (accession no. AJ132351; J.R. Stevens and W. Gibson, unpublished data), isolated from an Australian platypus (*Ornithorhynchus anatinus*) (Noyes *et al.*, 1999), and *T. therezieni* (Figure 4), the evolutionary consequences of which are discussed below.

The clear divergence of the aquatic clade from the salivarian trypanosomes

rules out Baker's earlier hypothesis that the leech-transmitted trypanosomes were ancestral salivarian forms (Baker, 1963). Indeed, the aquatic clade may be an early diverging branch from the *Trypanosoma* lineage as a whole, although the level of bootstrap support for the early divergence of this clade using ssu rRNA data by parsimony analysis is somewhat low (Lukeš *et al.*, 1997; Stevens *et al.*, 1999a; Figure 4) and by no means universal (Haag *et al.*, 1998; Stevens *et al.*, 1998).

The placing of *T. binneyi*, a trypanosome from a platypus, firmly in the aquatic clade with leech-transmitted trypanosomes from aquatic and amphibian hosts (Figure 4) is of interest in the context of host–parasite co-evolution. Such a result indicates that host ecology may sometimes play a greater role than host phylogeny in determining host–parasite associations and suggests that the platypus may have acquired its trypanosome species from another aquatic host. Similarly, the placing of *T. therezieni*, from a Madagascan chameleon, in the aquatic clade may represent another example of parasite host 'switching'. The phylogenetic relationships of *T. therezieni* are discussed in more detail by Haag *et al.* (1998).

4.4. The *Trypanosoma brucei* Clade

In early phylogenetic trees (e.g., those of Sogin *et al.*, 1986; Fernandes *et al.*, 1993; Maslov *et al.*, 1996) the only representative of the salivarian trypanosomes included was *T. brucei*, which appeared alone on a long, deep branch (Figure 3). Inclusion of further salivarian trypanosome species defined a *T. brucei* clade, as expected from the comparative biological data given above (Section 4.1, p. 11) (Haag *et al.*, 1998; Stevens *et al.*, 1999a; Figure 4). The clade consists of the salivarian tsetse-transmitted trypanosomes of African mammals. *T. evansi* and *T. equiperdum*, although not tsetse-transmitted and not restricted to Africa, also belong here by virtue of their close morphological and genetic similarity to *T. brucei*. Analysis of kinetoplast DNA (Borst *et al.*, 1987) and isoenzymes (Gibson *et al.*, 1983; Lun *et al.*, 1992) points to *T. evansi* and *T. equiperdum* being comparatively recent derivatives of *T. brucei*, which have been able to spread outside Africa because they no longer rely on tsetse transmission. Similarly, *T. vivax* has been imported into South America in the recent past.

The *T. brucei* clade is well separated from the rest of the trypanosome species, suggesting a distinct evolutionary history confined to Africa and associated with the tsetse fly. The position of *T. vivax* on the edge of the group is consistent with the comparative data discussed above. The subgeneric divisions are also supported by the phylogeny (Figure 4), although minor variations in the position of some terminal taxa are apparent. For example, other studies show *T. congolense* savannah and forest subgroups to be more closely related

to each other than to *T. congolense* from the Kenya coast (kilifi) (Garside and Gibson, 1995).

4.4.1. *Evolution of Virulence in* Trypanosoma brucei

The difference in virulence between *T. brucei gambiense* and *T. b. rhodesiense,* the two subspecies which cause human African trypanosomiasis (HAT; sleeping sickness) in tropical Africa, has been cited as an example of co-evolutionary attenuation of virulence in the past (reviewed by Toft and Karter, 1990). *T. b. rhodesiense* causes an acute form of HAT in East Africa, which has long been known to be a zoonotic disease carried by wild mammals. By contrast, *T. b. gambiense* causes a much less virulent, though still lethal, form of the disease in West and Central Africa. Although it is now known that this disease is also zoonotic, it was believed for many years that the human reservoir of infection alone was sufficient to maintain transmission. This led to the idea that *T. b. gambiense* had become gradually less virulent by co-evolution with its human host. However, ideas on the evolution of virulence have moved on (Ewald, 1983, 1995), and Toft and Karter (1990) dismissed this version of events as a 'just-so' story. Biochemical strain characterization has revealed that the avirulent *T. b. gambiense* is highly homogeneous throughout its range, while *T. b. rhodesiense* and also *T. b. brucei* are remarkably heterogeneous; for example, each epidemic focus of *T. b.rhodesiense* sleeping sickness appears to be associated with several different strains of the parasite. It is now realized that extensive gene flow may be occurring between *T. b. rhodesiense* and *T. b. brucei*, but not *T. b. gambiense* (see Gibson and Stevens, 1999) and this may be the mechanism by which heterogeneity is increased in *T. b. rhodesiense* relative to *T. b. gambiense*, with the corollary of reduced virulence in the latter (cf. the study on *T. cruzi* by Dias and Coura, 1997).

Recent results put a further twist on the evolution of *T. b. gambiense* and *T. b. rhodesiense*: Van Xong *et al.* (1998) identified a single gene from *T. b. rhodesiense*, which was capable of transforming the phenotype of a *T. b. brucei* clone from sensitivity to resistance to the lytic factor in human serum. Expression of this gene could not be detected in *T. b. gambiense*, however, suggesting that the trait for human infectivity in this subspecies may have a different basis. Further analysis of the mechanism of human infectivity in *T. brucei* spp. may yet yield clues to the evolution of *T. b. gambiense* and *T. b. rhodesiense.*

4.5. The *Trypanosoma cruzi* Clade

The *T. cruzi* clade, which includes the two human-infective species *T. cruzi* and *T. rangeli*, contains a range of species originating predominantly from South

American mammals. Interesting exceptions are three species of bat trypanosomes from Africa and Europe, and one as yet unnamed species of kangaroo trypanosome from Australia (Noyes *et al.*, 1999). The evolutionary significance of the composition of the *T. cruzi* clade is considerable and has been used to date major divergence events within *Trypanosoma* (Section 4.7.4, p. 27). Certainly, the inclusion of taxa from bats and South American mammals in recent studies (Figure 3; Stevens *et al.*, 1999a, b) has allowed clarification of the evolutionary relationships of *T. cruzi* and *T. rangeli*, revealing their apparently close evolutionary pathways and suggesting divergence from a common ancestor. The increased degree of definition afforded by an increased number of taxa indicates a very much more complex set of relationships among South American mammalian trypanosomes than previously supposed (Stevens *et al.*, 1999b).

4.5.1. *The Evolution of* Trypanosoma cruzi

Classically, *T. cruzi sensu lato* is divided into *T. cruzi cruzi* (hereafter referred to as *T. cruzi sensu strictu*) and *T. cruzi marinkellei*, a subspecies apparently confined to bats in South America (Baker *et al.*, 1978). Within *T. cruzi s.s.*, further subdivisions based on multi-locus isoenzyme profiles – zymodemes Z1, Z2, Z3 – have been defined (Miles *et al.*, 1977, 1978, 1980) with each zymodeme being associated with particular epidemiological characteristics and transmission cycles (Miles and Cibulskis, 1986). Greater clonal variability was seen by analysis of more isoenzyme loci (Tibayrenc *et al.*, 1986) and by analysis of restriction fragments of kinetoplast DNA (Morel *et al.*, 1980), DNA fingerprinting (Macedo *et al.*, 1995), and chromosome blotting (Henriksson *et al.*, 1993). Latterly, two distinct lineages within *T. cruzi s.s.* have been confirmed on the basis of molecular markers: random amplification of polymorphic DNA (RAPD) (Tibayrenc *et al.*, 1993; Tibayrenc, 1995; Souto *et al.*, 1996; Brisse *et al.*, 1998); miniexon gene sequences and 24S lsu rRNA sequences (Souto *et al.*, 1996; Zingales *et al.*, 1998; Fernandes *et al.*, 1998); cytochrome b sequences (Brisse, 1997) and the topoisomerase locus (Dos Santos and Buck, 1999). The genetic evidence, together with different pathological and geographical characteristics, strongly supports the division of *T. cruzi s.s.* into two main groups, recently denominated by international discussion (Anonymous, 1999) as *T. cruzi* 1 (corresponding to Z1 of Miles *et al.*, 1977, lineage 2 of Souto *et al.*, 1996) and *T. cruzi* 2 (corresponding to Z2 of Miles *et al.*, 1977, lineage 1 of Souto *et al.*, 1996). Of these, *T. cruzi* 1 seems the more homogeneous and, from studies throughout the Americas, seems primitively associated with opossums (*Didelphis* spp.). By contrast, *T. cruzi* 2 is more heterogeneous, with a number of well-characterized natural clones found mainly in 'southern cone'

countries, and seems primitively associated with rodents. *T. cruzi* 2 in human infections is also associated with chronic intestinal lesions (e.g. megaoesophagus, megacolon) as well as the cardiopathy that characterizes *T. cruzi* 1 infections. *T. cruzi* Z3 of Miles *et al.* (1981) is also highly heterogeneous and seems, by analysis of miniexon gene sequences, to have affinity with *T. cruzi* 2, although it can generally be recognized as a subclade characterized by a *c.* 50bp insertion in the non-transcribed spacer region of the miniexon (Fernandes *et al.*, 1998). It is almost invariably sylvatic, with current evidence suggesting a primitive association with armadillos. *T. cruzi marinkellei* (sometimes referred to as *T. cruzi* Z4) is strongly associated with bats.

Phylogenetic analysis based on ssu rRNA data (Figure 4) shows that *T. cruzi sensu lato* forms a discrete clade, within which the subspecies *T. c. marinkellei* appears somewhat distinct (68% bootstrap support – value not shown). However, due to the broad nature of the analysis presented in Figure 4 (which includes representatives of most major kinetoplastid groups), the alignment includes only nucleotide positions common to all the taxa (1809 nucleotides) and thus omits many sequence data capable of providing intraclade phylogenetic resolution (Stevens *et al.*, 1999a). Analysis of a subset of data, including all *T. cruzi s.l.* taxa with the bat trypanosome *T.* (*Schizotrypanum*) *dionisii* as an outgroup, provides a significant increase in phylogenetic resolution (Figure 5). This subtree suggests that the major phylogenetic groupings defined within *T. cruzi* have diverged at around the same time, and extended analysis of the work of Brisse (1997) on cytochrome b gene sequences suggests a divergence time of more than 5.5 million years between the most divergent lineages of *T. cruzi s.s.* (S. Brisse, personal communication), which is consistent with current biogeographical ideas about the evolution of the *T. cruzi* clade.

The original work by Souto *et al.* (1996) offered two hypotheses for the divergence of *T. cruzi* 1 and 2, in one of which *T. cruzi* 2 could have arisen by loss of rDNA cistrons from an ancestral form resembling *T. cruzi* 1. Their evidence, together with the relative homogeneity, wide distribution and close association with opossums, suggests that *T. cruzi* 1 can be considered the more primitive form – an idea strengthened by inclusion of the kangaroo trypanosome within the *T. cruzi* clade (Stevens *et al.*, 1999a). Moreover, infected opossums can maintain a patent parasitaemia throughout life with no apparent clinical symptom (Deane *et al.*, 1986), while *T. cruzi* can complete its entire development cycle in the anal glands of opossums and is readily transmitted in their anal gland secretions (Deane *et al.*, 1986). Opossum anal glands can also be infected with a range of other flagellates, giving rise to the superficial suggestion that *T. cruzi* may originally have been no more than a leptomonad that developed a complement-resistant stage enabling it to survive in the bloodstream as well as the immunologically privileged anal glands themselves (Schofield, 1987). It may be that the ancestor of the *T. cruzi* clade existed

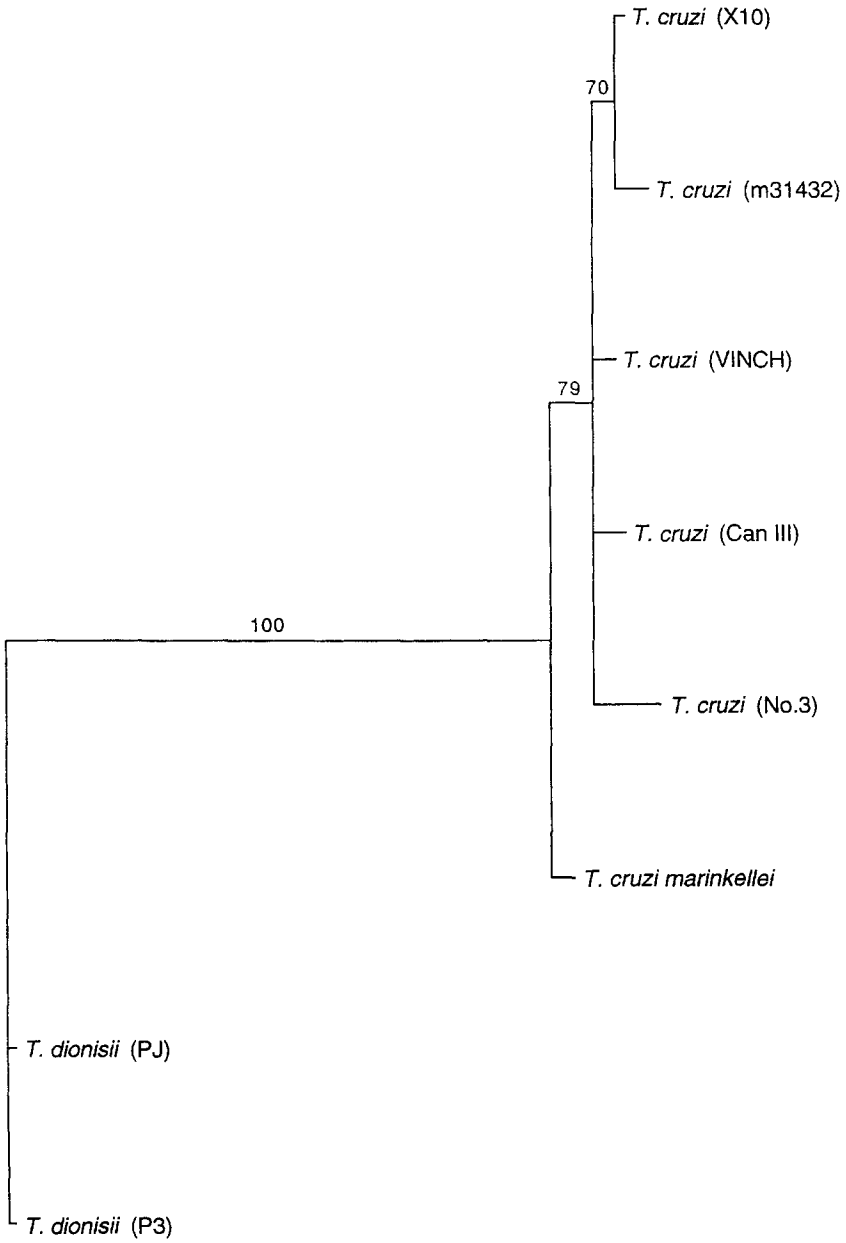

Figure 5 Phylogram constructed by bootstrapped (100 replicates) maximum likelihood analysis of 18S rRNA sequences of eight *T.* (*Schizotrypanum*) species or subspecies. The tree is based on an alignment of 2194 nucleotide positions, using *T. dionisii* as an outgroup (see Figure 4). Full details of taxa are given by Stevens *et al.* (1999a). Analysis was perfomed using PAUP* 4 (Swofford, 1998).

within the southern supercontinent some 80–50 mybp as a parasite of marsupials, which subsequently diversified in the Neotropics when the advent of Triatominae provided vectors able to pass the original *T. cruzi* from opossums into other mammalian hosts – particularly rodents, armadillos and bats. Certainly, evidence is accumulating to suggest that the insect vectors of *T. cruzi*, blood-sucking reduviids of the subfamily Triatominae, are relatively recent derivatives from a wide range of predaceous forms (Schofield, 1988). The Triatominae are clearly a polyphyletic assemblage, with tribes such as the Rhodniini and Triatomini having little in common beyond a general reduviid form combined with a series of convergent – but often functionally distinct – characters associated with the blood-sucking habit (Schofield, 1994, 1996). The major groupings of the Triatominae may well have arisen at different times, but none shows evidence of a long association with haematophagy and many retain facultatively predaceous habits – especially in the immature stages (Schofield and Dolling, 1993).

For the moment it is impossible to ascertain whether the ancestral *T. cruzi* was a monogenetic parasite transmitted directly between opossums in urine and anal gland secretions, or whether other vectors transmitted the parasites before the involvement of triatomines in the cycle. Alternatively, either one or both of the dates for the appearance of the *T. cruzi* clade and for the development of haematophagy in triatomines may be incorrect. Given the apparent age of the *T. cruzi* clade (50–80 million years) and the relatively recent advent of triatomines, it is evident that certain key aspects of *T. cruzi* evolution remain to be resolved.

The more recent radiation and spread of *T. cruzi s.s.* (and its subspecies) seem likely to have followed the emergence of the main lineages of Triatominae, followed by the spread of different host and vector species. The earliest divergence to *T. c. marinkellei*, indicated by the ssu rRNA data (Figure 5), would be associated with the Cavernicolini, species intimately associated with, bats that form one of the most distinctive and divergent tribes of the Triatominae. Divergence to the Z3 forms, which are found northwards from central Brazil, could be associated particularly with *Panstrongylus* – a South American genus of Triatominae that extends (as *P. rufotuberculatus* and *P. geniculatus*) into the southern part of Central America and is frequently found in armadillo burrows as well as opossum lodges. The Z3 forms could have been dispersed from South America by the armadillos and opossums themselves into North America, where they are now widespread (C. Barnabé and M. Tibayrenc, personal communication), subsequently encountering different triatomine vectors such as *Triatoma gerstaeckeri* and *Triatoma sanguisuga*.

Divergence to *T. cruzi* 2 is clearly more complex. Following the theories presented above, reinforced by the clear heterogeneity of *T. cruzi* 2, we propose that divergence to *T. cruzi* 2 would have occurred several times as the original opossum parasite was passed by different species of *Triatoma* into different

rodent hosts. Of particular interest is the possible fate of clonet 39 (Tibayrenc and Ayala, 1988), which is strongly associated with caviid rodents in Bolivia, with *Triatoma infestans* and with virulent human infections throughout much of the 'southern cone' region of South America. Historical reconstruction (Schofield, 1988), combined with multilocus isoenzyme electrophoresis (Dujardin, J.P. *et al.*, 1998) and karyotype analysis (Panzera *et al.*, 1998), strongly suggests that *T. infestans* originated in the Cochabamba/Sucre region of central Bolivia, from where it has been spread in association with human migrations, mainly over the last 100–150 years. Its arrival in central Brazil in the 1930s, and in north-eastern Brazil in the 1970s, is well documented and associated with outbreaks of acute Chagas disease in humans, invariably characterized as *T. cruzi* Z2 (e.g. Barrett *et al.*, 1976) and often as clonet 39 (Tibayrenc and Ayala, 1988). Nevertheless, several other clonets of *T. cruzi* 2 found in central Brazil are not necessarily found in central Bolivia, which suggests that many – if not all – of the recognized clonets of *T. cruzi* 2 may represent independent evolutionary events following parallel routes of adaptation into different rodent populations.

A further divergence can be suspected, which follows current understanding of the evolution of *Triatoma rubrofasciata*. This species of *Triatoma* is strongly associated with domestic rats, and has been found in port areas throughout the tropics and subtropics (Ryckman and Archbold, 1981), apparently spread in sailing ships from the New World to the Old along 17th–18th century trade routes (Gorla *et al.*, 1997). No sylvatic ecotope of this species has been reported, but morphological and morphometric analysis consistently place *T. rubrofasciata* with North American species of *Triatoma* such as *Triatoma sanguisuga* (J.P. Dujardin and C.J. Schofield, unpublished observations), which is frequently found in nests of pack rats (*Neotoma* spp.). *Triatoma rubrofasciata* is the principal vector of the rat trypanosome, *T. conorhini*, so that the phylogenetic position of *T. conorhini* within the *T. cruzi* clade (Figure 4) suggests that it may be a further specialized derivative from the putative *T. cruzi* ancestor.

4.5.2. *The Evolution of* Trypanosoma rangeli

The taxonomic and evolutionary status of *T. rangeli* has long been the subject of debate (Hoare, 1972; D'Alessandro and Saravia, 1992; Taverne, 1998; Stevens and Gibson, 1999). Unlike *T. cruzi*, *T. rangeli* produces only a transient and non-pathological infection in humans and other mammals such as opossums, but is frequently pathogenic to its triatomine bug vectors. In mammalian hosts *T. rangeli* multiplies as trypomastigotes in the bloodstream and it is not clear if tissue forms are normally produced [in the laboratory, amastigote-like forms of *T. rangeli* can be produced in a histiocytic cell line (Osorio *et al.*,

1995), but it is not known if such forms contribute to survival in mammalian hosts]. In the insect vectors, infective metacyclic forms develop in both anterior (salivary glands) and posterior (hindgut) sites, and transmission seems possible by either route (D'Alessandro and Saravia, 1992). On morphological and behavioural criteria, *T. rangeli* is generally classified within the subgenus *Herpetosoma* (see Hoare, 1972; D'Alessandro and Saravia, 1992), although Añez (1982) allied it to the Salivaria and argued for the creation of a new subgenus, *Tejeraia*.

In nature, *T. rangeli* seems associated primarily with species of *Rhodnius* and most reports of it being found naturally in species of *Triatoma* have been discounted. Even amongst species of *Rhodnius*, however, its behaviour is extremely variable, with some strains provoking very high mortality of some vector populations but not others. *T. rangeli* seems to share numerous immunological epitopes with *T. cruzi*, often resulting in cross reactions in serological tests, so that differential diagnosis of the two infections has been the subject of considerable study – especially in Colombia and Venezuela where both species circulate in similar habitats and co-infections, both of vectors and vertebrates, are common (e.g. Guhl *et al.*, 1987; Macedo *et al.*, 1995; Vallejo *et al.*, 1999).

Molecular studies have considerably clarified the systematic and evolutionary relationships of *T. rangeli*. Murthy *et al.* (1992) showed that the miniexon repeat units of *T. rangeli* and *T. cruzi* were substantially different in both size and sequence, and that the repeat unit could be used as a species-specific DNA probe for *T. rangeli*. Polymerase chain reaction (PCR) amplification, using both miniexon and kinetoplast DNA minicircle primers, has been used to distinguish sylvatic from domestic strains of *T. rangeli* in Colombia (Vallejo *et al.*, 1994, 1999). In Brazil, Steindel *et al.* (1998) examined *T. rangeli*, *T. cruzi* and a range of bat trypanosomes by isoenzyme and RAPD analysis and found they fell into three genetically distinct groups. The limited study of ß-tubulin gene sequences by Amorim *et al.* (1993) suggested that *T. rangeli* is more closely related to *T. brucei* than to *T. cruzi*, but this has not been confirmed by more broadly-based phylogenetic studies. For example, Stevens *et al.* (1999b) found that five *T. rangeli* isolates of diverse origins had almost identical ssu rRNA sequences and could be placed unequivocally in the *T. cruzi* clade by phylogenetic analysis. Furthermore, two related South American trypanosome species, *T. leeuwenhoeki* from a sloth and *T. minasense* from a squirrel monkey, also had identical ssu rRNA sequences to *T. rangeli*, suggesting that these species may be synonymous – although D'Alessandro and Saravia (1992) stated that *T. leeuwenhoeki* had a distinct isoenzymatic profile and did not develop in triatomines. The miniexon repeat unit sequences of *T. rangeli* and *T. leeuwenhoeki* were also highly homologous (*T. minasense* was not examined). Since both *T. rangeli* and *T. leeuwenhoeki* ostensibly belong to the subgenus *Herpetomonas*, and *T. minasense* to the subgenus

Megatrypanum, this result also indicates the need for revision of subgeneric classification in the Stercoraria (see Section 4.6, below).

Overall, results from a diverse range of phylogenetic studies indicate a close evolutionary relationship between *T. rangeli* and *T. cruzi*, probably within South America. These findings suggest that *T. rangeli* should be placed within the stercorarian subgenus *Schizotrypanum*, although it must be acknowledged that the whole basis for subgeneric classification within the Stercoraria section requires complete revision.

4.6. Phylogenetic and Taxonomic Anomalies

4.6.1. *Subgenus Classification*

The findings of molecular phylogenetic studies have begun to cast doubts on the evolutionary validity of some existing trypanosome taxonomy. In particular, within the Stercoraria, the integrity of the subgenera *Schizotrypanum*, *Herpetosoma* and *Megatrypanum* is now under question. For example, *T. (Megatrypanum) conorhini*, *T. (Megatrypanum) minasense*, *T. (Herpetosoma) leeuwenhoeki* and *T. (Herpetosoma) rangeli* all group with subgenus *Schizotrypanum* species in the *T. cruzi* clade (Stevens *et al.*, 1999a, b; Figure 4). Indeed, although the overall level of genetic diversity within the *T. cruzi* clade may not be as great as that observed in the *T. brucei* clade, the range of genetically distinct, specialist species present suggests a number of distinct evolutionary pathways within the group. This may eventually warrant recognition of more than one taxonomic division within the *T. cruzi* clade. As noted above (Section 4.5.2. p. 22), *T. rangeli* appears to have several synonyms, which are probably host-range variants.

In the study by Stevens *et al.* (1999b), species belonging to subgenera *Megatrypanum* or *Herpetomonas* were found in two or more phylogenetic groupings, indicating these subgenera to be polyphyletic. Certainly the subgenus *Megatrypanum*, as defined by Hoare (1972), contained an assortment of seemingly unrelated species and it is little surprise that it lacks evolutionary relevance. Other groupings emerging from phylogenetic analyses may in time serve as a basis for revision of subgeneric divisions within the genus *Trypanosoma*.

4.6.2. *Anomalous Specimens*

Single specimens are difficult to interpret until set in the context of a suitable body of data. In the case of trypanosomes, such a body of ssu rRNA sequence data now exists and, indeed, its existence is perhaps one of the main reasons for

the continued use of the ssu marker for phylogenetic studies. It can be seen that some single specimens have proved to be the key to interpreting the phylogenetic results (e.g. the kangaroo trypanosome isolate – see Section 4.7.4, p. 27), and there are several other apparently anomalous placements, which may have great evolutionary significance (Figure 4). For example, the placing of the platypus trypanosome *T. binneyi* (see Noyes *et al.*, 1999) and *T. therezieni* from a chameleon (Haag *et al.*, 1998) in the aquatic clade has already been mentioned (see Section 4.3, p. 15) and may provide insights into host–parasite co-evolution, the role of host ecology in determining the parasite complement of the host, and the frequency of host 'switching' (Lyal, 1986; Hafner and Nadler, 1988). Similarly, the placing of a trypanosome from an American kestrel (*T. benneti*, see Haag *et al.*, 1998) apart from other bird trypanosomes and the phylogenetic isolation of *T. grayi* (Figure 4) may indicate that these trypanosome species had very different evolutionary histories compared with other species from birds and reptiles.

The risk of laboratory error must always be borne in mind when attempting to interpret single anomalous specimens. The potential phylogenetic importance of some of the above single specimens simply underlines the need for more samples.

4.7. Dating the Phylogenetic Trees

4.7.1. *Molecular 'Clocks'*

Phylogenetic trees for trypanosomes are of interest for what they can reveal about the evolution of parasitism and other characteristics, such as antigenic variation, in the group. Interpretation of the tree in relation to other events on the evolutionary time scale depends on conversion of branch points into dates to estimate time of divergence of different clades. The molecular 'clock' approach (Zuckerkandl and Pauling, 1965) assumes that changes in a given sequence accumulate at a constant rate and, accordingly, that the difference between two sequences is a measure of the time of divergence. However, as our understanding of genome evolution grows, the concept of a uniform molecular 'clock' appears oversimplistic and indeed the approach has been amply criticized over the years (Fitch, 1976; Sibley and Ahlquist, 1984; Wilson *et al.*, 1987). Moreover, the idea of genomic evolution associated with an accumulation of mutations over time takes no account of factors such as founder effects and genetic drift, or recombination and intersibling competition, which probably feature strongly in the evolution of clonal organisms such as trypanosomes (Tibayrenc, 1995) and also in 'pseudoclones' such as domestic Triatominae (Schofield *et al.*, 1999). In the Triatominae at least, there is now strong evidence of morphologically distinct populations that appear genetically identical (e.g. Monteiro *et al.*, 1999) and the

converse of genetically distinct populations that appear morphologically identical (e.g. Dujardin, J.P. *et al.*, 1999a).

Thus, the divergence times of approximately 300 mybp, proposed by Haag *et al.* (1998) for the salivarian trypanosomes using an estimated 'clock' speed of 0.85% substitutions per 100 million years derived from ribosomal RNA analysis of metazoans (Escalante and Ayala, 1995), and of 340 mybp proposed by Fernandes *et al.* (1993) for the trypanosome and *Leishmania* lineages, are questionable. Nevertheless, within a given taxonomic group and well defined categories of genetic marker, the concept of a molecular 'clock' may be usefully employed for dating species divergence.

4.7.2. *Evolutionary Rates*

Recent studies of trypanosome phylogenies based on ssu rRNA have identified considerable differences in genetic diversity within clades in the genus *Trypanosoma*, focusing in particular on the apparently high rate of sequence evolution within *T. brucei* and related species (Lukeš *et al.*, 1997; Haag *et al.*, 1998; Noyes, 1998a; Stevens *et al.*, 1998, 1999a).

Lukeš *et al.* (1997) suggested that paraphyly of the genus *Trypanosoma*, as reported in a number of previous studies (e.g. that by Maslov *et al.*, 1996), may have been the result of a high rate of nucleotide substitutions in the *T. brucei* and outgroup lineages and an associated high level of homoplasy in the ssu rRNA sequences of *T. brucei* and outgroup species. Similarly, the study by Haag *et al.* (1998) identified several-fold substitution rate differences between clades, with the rate in *T. vivax* being up to three times that in lineages leading to certain non-salivarian taxa. Moreover, Haag *et al.* (1998) identified salivarian trypanosomes as generally more distant from (two) outgroup species than were other taxa while, within the Salivaria, *T. vivax* was consistently more distant from the outgroup taxa (by several percent of sequence divergence) than were other salivarian species. Indeed, to reduce possible long branch effects associated with such divergent taxa, Haag *et al.* (1998) performed their analyses excluding various rapidly evolving lineages, e.g. *T. vivax*, while confirming the monophyly of the genus.

More recently, Stevens *et al.* (1998) and Noyes and Rambaut (1998) have identified differences in intra-clade evolution rates of approximately eight-fold, based on comparisons of deeper branch lengths within the salivarian clade and the *T. cruzi* clade (Figure 4). The exact extent to which the rapid evolution of certain lineages within the salivarian clade may have distorted the topology of the tree (and hence the estimates of evolutionary rate) is unknown but, as identified by Haag *et al.* (1998), significant differences exist even between taxa within the same clade, e.g. *T. vivax* and other Salivaria. Latterly, work by J.R. Stevens and A. Rambaut (unpublished) has focused on

identifying the different rates at which trypanosomes are evolving; preliminary results suggest that more than three different rate parameters are required to explain phylogenetic relationships within the genus *Trypanosoma*.

Nevertheless, despite the existence of significant rate differences within trees, more recent phylogenies based on ssu rRNA do now appear sufficiently robust to have avoided the Salivaria being drawn towards outgroup taxa by the phenomenon of long-branch attraction (Felsenstein, 1978, 1988; Hendy and Penny, 1989), a problem encountered in many previous studies (e.g. Fernandes *et al.*, 1993 and Maslov *et al.*, 1994, 1996).

4.7.3. *Host–Parasite Co-evolution*

A second method by which divergence times can be estimated relies on congruence of host and parasite phylogenies. Parasite trees can be calibrated by reference to known time points within host phylogenies, which have been dated by independent methods, e.g. the fossil record. Using this approach, the divergence of fish from higher vertebrates (400 mybp) and the divergence of birds from rodents (220 mybp) have been used to estimate the split of salivarian trypanosomes from other trypanosomes as occurring 260 and 500 mybp, respectively (Haag *et al.*, 1998). On a geological time scale, even the most recent host–parasite-based estimate of 260 mybp places the divergence of the Salivaria in the Permian, at a time when reptiles were the dominant group of vertebrates. If correct, such a date suggests that the Salivaria would have diverged long before even the most primitive ancestors of their present vertebrate and invertebrate hosts had appeared. The use of such an approach assumes, of course, that existing associations of hosts and parasites reflect past relationships. However, while such relationships are generally assumed to have arisen as a result of uninterrupted association (Hafner and Nadler, 1988), host 'switching', sometimes referred to as host colonization, may also have disrupted the relationship between host and parasite phylogenies (Mitter *et al.*, 1991). Certainly, such host 'switching', as evidenced in the current study by the apparently close evolutionary relationship of a trypanosome from a platypus with other trypanosomes from aquatic hosts, does appear to have occurred. Such a phenomenon may perhaps at least partly explain the two very different estimates of divergence times obtained by Haag *et al.* (1998) using this approach.

4.7.4. *Vicariance Biogeography*

Perhaps, by considering trypanosome phylogeny in the context of known biogeographical events, a more realistic estimation of divergence could be

obtained. This approach to phylogenetic calibration is known as vicariance biogeography (Wiley, 1988) and several studies of trypanosomatids have drawn on this technique, for example using the separation of Africa and South America to date the divergence of *Leishmania* and *Trypanosoma* (Lake *et al.*, 1988), to corroborate the split between Old and New World species of *Leishmania* (see Fernandes *et al.*, 1993) and, most recently, to date the divergence of *T. brucei* and *T. cruzi* (see Stevens *et al.*, 1999a). From this latter study, the divergence of the salivarian clade from other *Trypanosoma* spp. is dated to the mid-Cretaceous period, around 100 mybp, when Africa became isolated from the other continents (Parrish, 1993; Smith *et al.*, 1994). This is based on the observations that the *T. brucei* clade consists exclusively of African mammalian tsetse-transmitted species and that trypanosome species from African amphibia and reptiles are unrelated (*T. mega*, *T. grayi*, *T. varani*; Figure 4). At this time, the ancestors of most extant mammalian groups were present but had not yet begun major diversification and it is easy to envisage subsequent co-evolution of this clade with African hosts. Interestingly, Lambrecht (1980) arrived at a similar evolutionary scenario considering only palaeoecological data.

The composition of the *T. cruzi* clade – predominantly mammalian trypanosome species from South America – also agrees with this interpretation and, significantly, the inclusion of an Australian marsupial trypanosome in the clade (*Trypanosoma* sp. H25 kangaroo, Figure 4) reinforces the idea that this grouping had its origin on a southern super-continent of South America, Antarctica and Australia, which remained linked together after the separation from Africa (Cox and Moore, 1993). Furthermore, the only trypanosomes from this clade found in the Old World are those infecting bats, mammals that are able to fly across geographical barriers. This finding is further supported by the recent work of Stevens *et al.* (1999b; see also Figure 4), in which a trypanosome from an African bat is also classified unequivocally with other *T. cruzi* clade taxa, together with a range of new sequences from trypanosomes from a variety of South American mammals. The alternative interpretation that *T. cruzi* evolved in bats and spread with them to South America is unlikely since, in the Old World, subgenus *Schizotrypanum* trypanosomes are, as far as is known, restricted to bats (Hoare, 1972; Baker *et al.*, 1978).

The early evolution of the *T. cruzi* clade seems most likely to have been associated with the dominant marsupial fauna of the region. The neotropical opossums, Didelphidae, usually make dens in tree hollows, where they can act as hosts for triatomines. Kangaroos were also primitively arboreal (Szalay, 1994) and therefore may have shared the same environment, if not niche, with ancestors of the Didelphidae. Marsupials first appeared in Gondwana at the end of the Cretaceous and underwent a rapid adaptive radiation approximately 70 mybp, arriving in Australia by about 56 mybp (Springer *et al.*, 1997). Australia

finally became separated from the Neotropics about 50 mybp, although the passage through Antarctica may have been difficult for some time before that. Consequently, from the inclusion of the kangaroo trypanosome in the *T. cruzi* clade we can postulate that this clade arose 80–50 mybp, and perhaps earlier rather than later in that period.

4.7.5. *Evolutionary History of Human* Trypanosomiases

Regardless of the actual date of divergence of *T. brucei* and *T. cruzi*, it is clear from the phylogenetic evidence that these two pathogens of humans developed according to different patterns over very different time scales. In Africa, *T. brucei* appears to have shared a long period of co-evolution with primates (~15 million years) and the genus *Homo* (~3 million years; Lewin, 1993), presumably in continuous contact with tsetse flies (Jordan, 1993). Taking the example of malaria, where several mechanisms of genetic resistance have been selected in the susceptible human population, a prolonged period of struggle between trypanosome and host should also have led to selection for increased host defences. It is tempting to speculate that the long evolutionary history of humans with salivarian trypanosomes explains our present innate resistance to infection with most species of tsetse-transmitted trypanosome by virtue of a trypanolytic factor in the serum, a trait shared with baboons, gorillas and mandrills (Hawking *et al.*, 1973; Seed *et al.*, 1999).

In contrast, human contact with *T. cruzi* and *T. rangeli* would not have occurred before human migration to the Americas, which is generally dated no earlier than 30 000–40 000 years ago; indeed, there is no evidence for contact earlier than 3000 years before present when early settlements were made by previously nomadic cultures in northern Chile (Rothhammer *et al.*, 1985). A high proportion of the mummified bodies from these pre-Colombian settlements show clinical signs consistent with a diagnosis of chronic Chagas disease and *T. cruzi* DNA has now been isolated from some of the mummified tissue samples (Guhl *et al.*, 1997). Interestingly, there is good clinical evidence to suggest that human infection with *T. cruzi* in Chile now produces a relatively benign disease compared with the virulent infections seen in Argentina and Brazil, where the human disease has been known only since the latter half of the 19th century. The indigenous peoples of northern Chile also have a higher frequency than Europeans of human leucocyte antigen types that are protective against chagasic cardiac abnormalities (Llop *et al.*, 1988). In Argentina, Paraguay and Brazil, there is also evidence to suggest that the virulence of human infections has been declining since the middle of this century – shown especially by a marked reduction in the clinical symptoms of acute infections (Dias and Coura, 1997).

Questions concerning the nature of such evolution, however, remain to be answered. The existence of genetic exchange between trypanosomes in tsetse flies is now well recognized (Jenni *et al.*, 1986; Gibson, 1995) and its possible effect on the history of disease evolution cannot be discounted. In South America, however, the situation is less clear cut. Despite considerable evidence from population genetics studies suggesting that *T. cruzi* is a basically clonal organism (e.g. Tibayrenc *et al.*, 1990) which may have undergone infrequent genetic exchange at some time in its evolutionary history (the last time being at least 5.5 mybp; Brisse, 1997), results from recent isoenzyme-based studies of well-defined foci have identified hybrid patterns concordant with the products of genetic exchange (Bogliolo *et al.*, 1996; Carrasco *et al.*, 1996).

4.8. Outlook

Additional gene markers, with different levels of phylogenetic resolution, will undoubtedly help to unravel the higher level polytomy within *Trypanosoma* apparent in even recent phylogenies based on ssu rRNA gene analyses (e.g. Figure 4). Despite the inclusion of increasing numbers of species, work by Lukeš *et al.* (1997) and Stevens *et al.* (1998) indicates the sensitivity of such trees to different outgroup taxa and the effect on tree topology; such a finding may also have implications for the suitability of parsimony for analysing these data. The addition of two ssu rRNA sequences from *Phytomonas* sp. acted to reduce phylogenetic definition within the upper level of the genus *Trypanosoma*, so that the aquatic clade no longer diverged earlier than other *Trypanosoma* spp. (Stevens *et al.*, 1998). Interestingly, the phylogenetically difficult nature of *Phytomonas* has been highlighted in a number of other studies (Marché *et al.*, 1995; Hannaert *et al.*, 1998); for example, difficulties in resolving the relationship of the *Phytomonas* and *Herpetomonas* lineages by a variety of tree reconstruction methods were reported by Hannaert *et al.* (1998).

Thus, while it now seems certain that the genus *Trypanosoma* is monophyletic, it seems equally certain that the taxonomic status of the genus will not be fully resolved until the phylogenetic relationships of various closely related sister genera are also resolved. Similarly, as illustrated by recent studies of *T. rangeli*, based on ssu rRNA sequences (Stevens *et al.*, 1999b) and *T. cruzi*/*T. c. marinkellei*, based on mitochondrial cytochrome b sequences (Brisse, 1997), a range of important taxonomic and evolutionary questions relating to species within the genus remains to be answered.

5. GENUS *LEISHMANIA*

5.1. Overview

The genus *Leishmania* appears much more homogeneous than the genus *Trypanosoma.* For example, genetic diversity within the genus *Leishmania s.s.* is similar to that within the single trypanosome species *T. cruzi s.s.* (Tibayrenc *et al.*, 1993) and morphological differences between *Leishmania* species are difficult to detect. Nevertheless, the genus *Leishmania* contains approximately 30 species of parasites that infect mammals (Shaw, 1994) and 17 species that infect reptiles (Telford, 1995). The mammalian species are

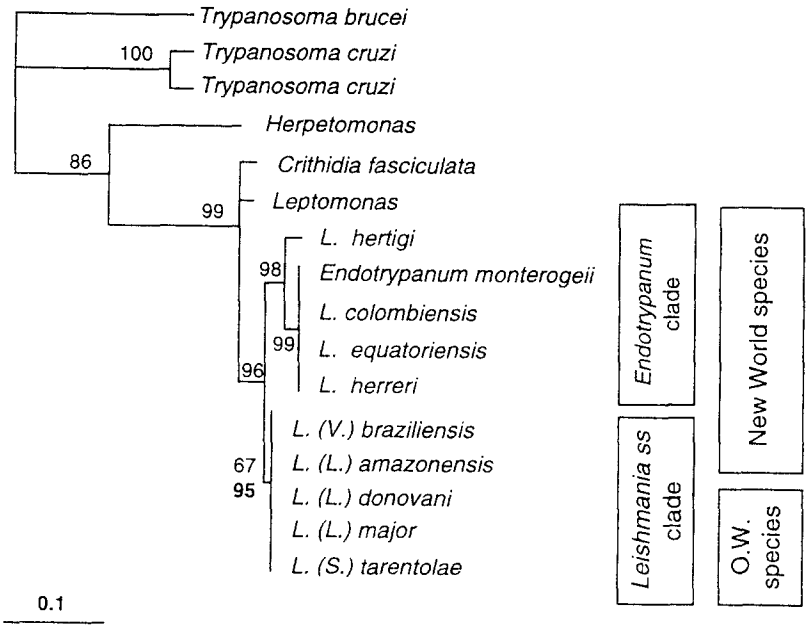

Figure 6 Phylogeny constructed by maximum likelihood analysis of partial 18S ssu rRNA gene sequences of *Leishmania* and *Endotrypanum* species, as described by Noyes *et al.* (1997), with the addition of *L. colombiensis* and *L. equatoriensis* sequences (GenBank accession nos. AF133836 and AF133837, respectively); analysis was performed using the program DNAML (which does not assume a molecular clock) in PHYLIP (Felsenstein, 1993). Scale bar denotes expected numbers of substitutions averaged over all sites analysed (see DNAML documentation). The same topology was also found by parsimony analysis (DNAPARS). Bootstrap values, under maximum likelihood (values shown; DNAML) and parsimony, are within 5% of each other, except for the *Leishmania s.s.* clade, support for which was 28 points higher by parsimony (shown in bold type). O.W. = Old World.

divided into two subgenera, *L. (Viannia)* which is restricted to the New World and *L. (Leishmania)* which is found in both the Old and New Worlds (Lainson and Shaw, 1987). There is a single subgenus containing the reptilian species, *L. (Sauroleishmania)*, which is restricted to the Old World (Telford, 1995; Noyes *et al.*, 1998).

It has recently been found that four *Leishmania* species – *L. herreri*, *L. hertigi*, *L. colombiensis* and *L. equatoriensis* – are more closely related to members of the sister genus *Endotrypanum* than to other members of the genus *Leishmania* (Figure 6; Noyes *et al.*, 1996b, 1997; Cupolillo *et al.*, 1998). The term *Leishmania sensu stricto* (*Leishmania s.s.*) will therefore be used to refer to all *Leishmania* species except the above-mentioned four. The sister clade to *Leishmania s.s.* is best known for the *Endotrypanum* parasites of sloths; this clade contains an increasingly heterogeneous assemblage of parasite species (Figure 6), the majority of which, as noted above, are *Leishmania* species. Although a revision of the nomenclature of this clade is clearly required, acquisition of further data on the anomalous species that it contains is necessary before this can be done with confidence. This clade, including the *Leishmania* species, will be referred to as the *Endotrypanum* clade to distinguish these species from *Leishmania s.s.*, although it may eventually be appropriate to include all these parasites within the genus *Leishmania*.

Although *Leishmania* parasites are very similar morphologically, they cause a wide range of pathologies in humans, ranging from subclinical infections through self-limiting cutaneous lesions to chronic mucocutaneous lesions and life-threatening visceral disease. There is a tendency for particular pathologies to be associated with particular species or groups of species of *Leishmania*. This has led to an intense search for molecular markers both to identify *Leishmania* strains and species and to find pathognomonic markers. Many of these data have also been used to classify groups of *Leishmania* species at various levels.

Until intrinsic characters such as isoenzymes and DNA buoyant densities became available, new *Leishmania* species were created primarily on the basis of extrinsic characters such as pathology in humans, geographical distribution, host specificity and development in sandflies and laboratory animals. Morphological and immunological characteristics were usually only secondary supporting characters (Lainson and Shaw, 1987).

During the 1980s the *Leishmania* species of lizards were placed in a new genus, *Sauroleishmania*, which was also primarily based on the use of extrinsic characters (Killick-Kendrick *et al.*, 1986). Although the genus *Sauroleishmania* was widely accepted by investigators with interests in the ecology and epidemiology of *Leishmania*, it was not accepted by those who were using the lizard *Leishmania* species as model organisms for studies of the biochemistry and genetics of *Leishmania* (see Telford, 1995; Thiemann *et al.*,

1998). More recent data have shown that the lizard species of *Leishmania* are very similar to those of mammals (van-Eys *et al.*, 1992; Previato *et al.*, 1997) and phylogenies of the ssu rRNA, DNA polymerase and RNA polymerase genes indicate that the lizard *Leishmania* species are derived from the mammalian forms (Croan *et al.*, 1997; Noyes *et al.*, 1997). It was therefore proposed that the lizard *Leishmania* species should be classified in a separate subgenus, *L.* (*Sauroleishmania*), as originally proposed by Ranque (cited by Noyes *et al.*, 1998), and this nomenclature will be used here.

The view of the lizard *Leishmania* as being quite distinct parasites guided the hypotheses of the evolution of *Leishmania* proposed by Saf'janova (1986) and Lainson and Shaw (1987). Saf'janova (1986) proposed that lizard and mammalian *Leishmania* had independent origins during the Mesozoic from *Leptomonas*-like parasites of primitive sandflies. The two subgenera of mammalian parasites were regarded as having been separated by continental drift at the end of the Mesozoic and it was suggested that *L. (Sauroleishmania)* developed only in the Old World because the sandfly vectors of these parasites, which are members of the genus *Sergentomyia*, are restricted to the Old World (Saf'janova, 1986). Lainson and Shaw (1987) also assumed a mid-Mesozoic origin of lizard *Leishmania*; however, they proposed that the mammalian *Leishmania* evolved from the reptilian forms rather than independently from *Leptomonas*-like parasites of sandflies. The subgenus *L. (Viannia)* of the New World was regarded as having a more primitive type of development in the posterior part of the sandfly gut than that of *L.* (*Leishmania*), which develops in the sandfly mid- and foreguts. This led Lainson and Shaw (1987) to suggest that mammalian *Leishmania* might have originally arisen in the Neotropics, possibly in edentates which are the most primitive order of eutherian mammals and which are particularly associated with *Leishmania* in the Neotropics; *L.* (*Leishmania*) would then have evolved from *L.* (*Viannia*)-like parasites and spread to the Old World in more recent times. This hypothesis for the origin of the mammalian *Leishmania* was later supported by the findings of molecular-based studies (Croan *et al.*, 1997; Noyes *et al.*, 1997).

5.2. Molecular and Biochemical Characterization Methods

Since the genus *Leishmania* is more homogeneous than *Trypanosoma*, techniques that reveal genetic variation at a higher level of resolution are required for phylogenetic analysis of *Leishmania* species. Thus, isoenzyme and restriction fragment length polymorphism (RFLP) analyses (which, for trypanosomes, are essentially population genetics techniques; see, for example, the work of Gibson and Stevens, 1999) are included here, but not in the previous section on trypanosomes.

5.2.1. *DNA Buoyant Density*

Quantitative methods suitable for the identification and classification of *Leishmania* species first became available in the 1970s. However the earliest methods were not entirely suitable for the classification of the whole genus or were not used effectively because, due to the ideas prevailing at the time, the reptilian species of *Leishmania* were excluded from these studies.

The buoyant density of kinetoplast and nuclear DNA was the first DNA-based method to be applied to the Kinetoplastida (Newton and Burnett, 1972), and to *Leishmania* (Chance *et al.*, 1974). DNA buoyant density is a measure of the overall guanine + cytosine content of an organism and, being a relatively low resolution technique, is suitable only for identifying the major *Leishmania* species complexes. In the original paper by Chance *et al.* (1974), the buoyant density values for a wide range of *Leishmania* species were included. These data allowed the identification of subsequently isolated strains but, since this method requires large numbers of parasites (10^9) and an expensive analytical centrifuge, it never came into widespread use. Nevertheless, DNA buoyant density is still the only measure of a whole genome character that has been applied to *Leishmania* parasites and provides valuable data for the investigation of the evolution of the genus *Leishmania*. The increase in buoyant density of nuclear and kinetoplast DNA approximately follows the branching order of the DNA/RNA polymerase phylogeny (Figure 7). The value of this character, however, for both the classification and identification of *Leishmania* spp. was not apparent until additional methods became available to support the surprising implications of these early data (Noyes *et al.*, 1997).

5.2.2. *Restriction Fragment Length Polymorphism Analysis*

RFLPs of genomic DNA were tested for suitability for evolutionary studies and it was found that the major species complexes could be identified by this method (Beverley *et al.*, 1987). However, there was too much variation to resolve the relationships between the complexes using the three probes and six restriction enzymes tested.

5.2.3. *Multilocus Isoenzyme Electrophoresis*

Multilocus isoenzyme electrophoresis (MLIE) was introduced during the 1970s and became the method of choice for identifying *Leishmania* species (Chance, 1985). In contrast to DNA buoyant density, MLIE is a very high resolution technique that has revealed considerable variation within the majority

of *Leishmania* species. As for bacteria and *Trypanosoma*, different isolates that have exactly the same mobilities for the isoenzymes studied form a zymodeme. The majority of *Leishmania* species that had been defined on extrinsic criteria were found to belong to well-defined groups of zymodemes. However, it became clear that individual *Leishmania* isolates could not be identified by pathology alone, since parasites causing similar pathologies were found to be members of different species by MLIE. Many *Leishmania* species and even individual zymodemes were found to cause a wide spectrum of clinical symptoms, such as *L. infantum* zymodeme MON-1 which is the most common cause of visceral leishmaniasis in the Mediterranean and South America but is also commonly isolated from cutaneous lesions (Moreno *et al.*, 1986).

Many studies have used MLIE to identify or classify particular groups of *Leishmania* parasites, and some have included an evolutionary interpretation. Of these, the first large-scale classification of the Old World mammalian *Leishmania* was conducted by Rioux *et al.* (1990) and this was later extended to include the New World species (Thomaz-Soccol *et al.*, 1993a, b). These phylogenies were interpreted as support for Saf'janova's (1986) hypothesis of a Mesozoic origin of the genus in Gondwana, separation of the two mammalian subgenera by continental drift, and subsequent migration of *L.* (*Leishmania*) from the Old World to the New, possibly with the caviomorph rodents.

However, these studies excluded the subgenus *L.* (*Sauroleishmania*), which at that time was considered to be a separate genus, and did not include an outgroup, since the appropriate outgroup was not then known. Moreover, as illustrated by the MLIE phylogeny proposed by Cupolillo *et al.* (1994), which differed in a number of significant respects from those of Thomaz-Soccol (1993a, b), these studies were working at the limit of MLIE resolution.

5.2.3. *Additional Molecular Analyses*

During the 1990s, various DNA based methods were applied to the identification of *Leishmania*, e.g. RAPD analysis, PCR amplification of miniexon DNA, and RFLP analysis of ribosomal intergenic spacers (Tibayrenc *et al.*, 1993; Cupolillo *et al.*, 1995; Noyes *et al.*, 1996a; Ramos *et al.*, 1996). However, none of these was used for classification of the entire *Leishmania* genus and only pulsed-field gel electrophoresis (PFGE) data have been used to formulate hypotheses for the evolution of groups of *Leishmania* parasites. Dujardin, J.C. *et al.* (1993, 1995) used the great variability of *Leishmania* karyotypes to examine the evolution of *Leishmania* populations from contiguous but isolated valleys in the Andes. The results suggested that *L.* (*V.*) *peruviana* had evolved from *L. (V.) braziliensis* strains at the forest boundary and spread progressively through the Andean valleys.

5.3. ssu rRNA Phylogenies

Phylogenies of the genus *Leishmania* based on gene sequencing, with resolution suitable for the classification of the *Leishmania* species complexes, took a relatively long time to appear. In general, the most widely used genes in molecular phylogenies are the ssu rRNA gene and mitochondrial gene sequences. Extensive editing of some mitochondrial genes in *Leishmania* (see Maslov *et al.*, 1994) means that considerable caution must be exercised when using these genes for phylogenetic analysis. Large numbers of partial sequences of the ssu rRNA gene were collected by van-Eys *et al.* (1992), and Fernandes *et al.* (1993) used the sequence of the ssu rRNA and lsu rRNA genes to demonstrate that the outgroup for the genus *Leishmania* was the genus *Endotrypanum*.

However, there was insufficient variation in the ssu rRNA gene to resolve any of the groups within *Leishmania*, although some *Leishmania* species complexes could be identified by RFLP analysis of the PCR product (van-Eys *et al.*, 1992). Accordingly, Noyes *et al.* (1997) used RFLP analysis of the complete ssu rRNA gene to prepare the first classification of the genus *Leishmania* that included both the lizard *Leishmania* and the genus *Endotrypanum* as an outgroup. This classification contained two surprising features: first, it suggested for the first time that the lizard *Leishmania* might be derived from mammalian parasites and not vice versa, as had been thought previously; secondly, the RFLP classification indicated that the *L. hertigi* complex was more closely related to the genus *Endotrypanum*, which is a sister genus of *Leishmania*, than to *Leishmania* itself. In this classification both the outgroup taxa and the *Leishmania* subgenus closest to the root of the tree were found only in the New World, whilst the crown of the tree was composed of the Old World subgenera *L. (Leishmania)* and *L. (Sauroleishmania)*. This led the authors to propose that the *Leishmania/Endotrypanum* clade had evolved from monogenetic parasites of sandflies in the New World during the early Cenozoic and had migrated to the Old World no later than the mid-Miocene. It was suggested that the reptilian *Leishmania* species had evolved in the Old World from these immigrant mammalian parasites.

Since it had been impossible to distinguish between the *Leishmania* complexes on the basis of the sequence of the ssu rRNA gene, it was surprising that RFLP-based classification could give a reliable result. However, the RFLP-based classification of Noyes *et al.* (1997) was supported by kinetoplast DNA cross-hybridization studies and by published DNA buoyant density measurements; a close relationship between the subgenus *L. (Sauroleishmania)* of lizards and mammalian *Leishmania* species was also supported by a number of other studies (Gomez-Eichelmann *et al.*, 1988; Briones *et al.*, 1992; Previato *et al.*, 1997). The strongest evidence for a Neotropical origin of the *Leishmania/Endotrypanum* clade came from

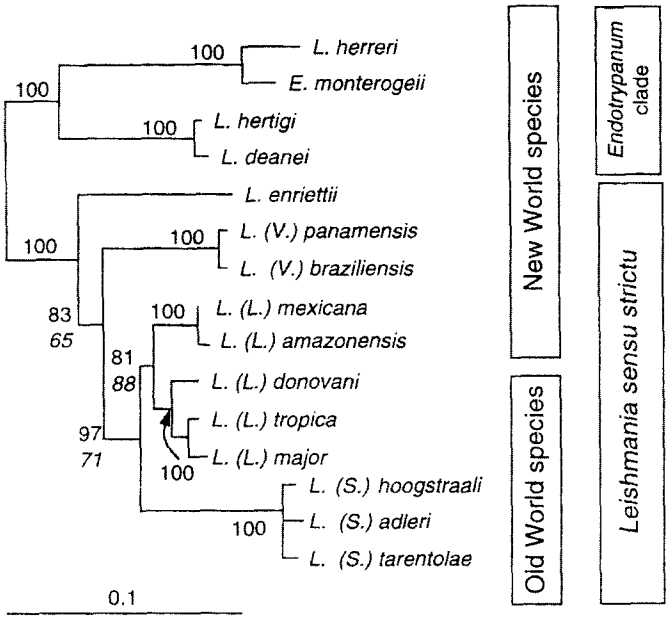

Figure 7 Phylogeny constructed by maximum likelihood analysis of partial DNA polymerase and RNA polymerase gene sequences of *Leishmania* and *Endotrypanum* species; analyses were performed using the program DNAML in PHYLIP (Felsenstein, 1993). The phylogeny was artificially rooted on the mid-point using RETREE; this mid-point root is supported by the position of the root of the *Leishmania/Endotrypanum* clade in the partial 18S rRNA phylogeny (Figure 6). Sequences are taken from the work of Croan *et al.* (1997), except for the *L. enriettii* sequences (H.A. Noyes, unpublished data; accession nos. AF151727 [RNA polymerase] and AF151728 [DNA polymerase]. Parsimony analysis of the same sequence alignment (DNAPARS) produced the same tree topology, but with some reduction in bootstrap values; where between-method bootstrap differences were >5%, the value obtained by parsimony analysis is also given in italics. The RNA and DNA polymerase phylogeny has the same topology as that given by Croan *et al.* (1997); all the most basal species are Neotropical, whilst only derived species are found in the Old World. The inclusion of *L. enriettii* further increases the diversity of the Neotropical *Leishmania* species relative to the Old World species and suggests that cladogenesis was occurring within the genus *Leishmania s.s.* in the Neotropics before any of the Old World species had arisen.

phylogenies of the RNA and DNA polymerase genes. Earlier studies of a 502 bp sequence of the RNA polymerase gene had indicated that lizard *Leishmania* were external to the mammalian *Leishmania*, but with low bootstrap support (54–75%; Croan and Ellis, 1996). However, analysis of a 1306 bp fragment of the RNA polymerase gene together with a 957–970 bp fragment of the DNA polymerase gene showed that the lizard *Leishmania* had arisen after *L. (Viannia)* of New World mammals (Croan *et al.*, 1997; Figure 7). This phylogeny also suggested that these genes were evolving faster in the lizard *Leishmania* than in the mammalian *Leishmania*, making it difficult to be confident of the position of the lizard parasites. Two consensus trees placed the lizard *Leishmania* external to the subgenus *L. (Leishmania)* and one consensus tree placed the lizard *Leishmania* between the New and Old World representatives of *L. (Leishmania)*. The subgenus *L. (Sauroleishmania)* may be evolving rapidly to adapt to its new class of vertebrate hosts. If the polymerase genes of *L. (Sauroleishmania)* are also evolving rapidly relative to *L. (Leishmania)*, the unstable classification of the subgenus *L. (Sauroleishmania)* in the polymerase phylogenies may be due to long-branch attraction (Felsenstein, 1978, 1988; Hendy and Penny, 1989). If the hypothesis of a Neotropical origin of *Leishmania* were correct, the true phylogeny would be the latter, which places the lizard *Leishmania* as a sister clade of the Old World *L. (Leishmania)* and not as a sister clade of the whole *L. (Leishmania)* subgenus.

5.4. DNA and RNA Polymerase Phylogenies

A combined phylogeny of the DNA and RNA polymerase gene sequences of Croan *et al.* (1997), with the addition of the sequences for *L. enriettii* (H.A. Noyes, unpublished data; RNA polymerase: AF151727; DNA polymerase: AF151728), is shown in Figure 7. This phylogeny has the same topology as that presented by Croan *et al.* (1997). Although little is known of the ecology of *L. enriettii* it has always been considered to be an uncontroversial member of the *L.* (*L.*) *mexicana* complex (Lainson and Shaw, 1987; Lainson, 1997). Consequently, it was surprising to find that *L. enriettii* was the most external member of the genus *Leishmania s.s.* in the DNA and RNA polymerase maximum likelihood phylogenies. Bootstrap support for this observation was 83% and 65% (Figure 7), depending on methodology, and thus *L. enriettii* appears to be only distantly related to the *L. (L.) mexicana* complex. This finding is further supported by the common absence of the gene *gp46* from *L. enriettii* and the subgenus *L. (Viannia)*, although it is present in *L. (Leishmania)* spp. (Hanekamp and Langer, 1991; McMahon-Pratt *et al.*, 1992). Since *L. enriettii* appears to be a parasite of caviomorph rodents, the finding that this parasite is the most external member of the genus *Leishmania s.s.* suggests that the genus

Leishmania and the genus *Endotrypanum* might have separated after the caviomorph rodents arrived in the Neotropics, around the Eocene–Oligocene boundary (35 mybp) (Flynn and Wyss, 1998). Alternatively, the position of *L. enriettii* external to the subgenus *L. (Viannia)* may be an artefact of an increased evolutionary rate in *L. enriettii*. If this were the case, the subgenus *L. (Viannia)*, which is particularly associated with edentates, would be the earliest branch of the *Leishmania* clade; the genus *Leishmania* and the genus *Endotrypanum* may then have arisen due to the separation of two groups of

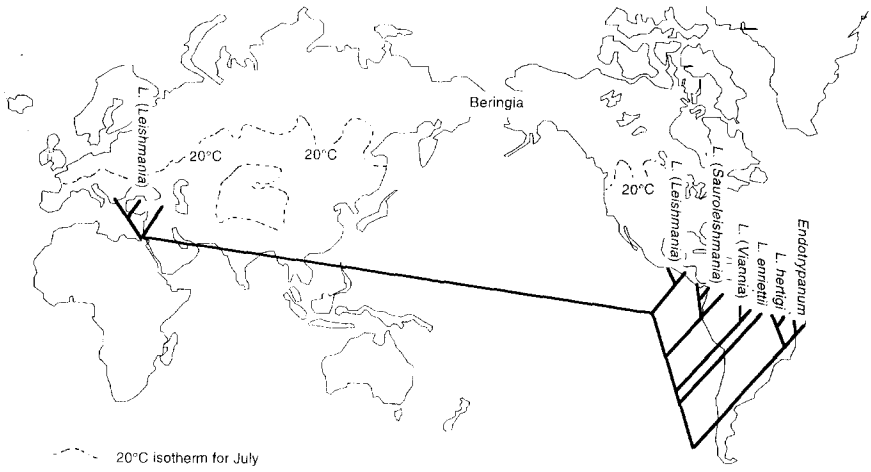

Figure 8 World map with the DNA and RNA polymerase gene phylogeny (Figure 7) superimposed, showing that the three most basal clades of this gene phylogeny are found in the Neotropics. The discordance between this phylogeny and the distribution of these clades is apparent in the position of the *L.* (*Sauroleishmania*) clade which is shown in the New World although it is found only in the Old World. This suggests either that the *L.* (*Sauroleishmania*) clade has been drawn towards the outgroup taxa by a faster rate of evolution or the *L.* (*Sauroleishmania*) clade first arose in the New World and then migrated to the Old World, before becoming extinct in the New World. The first hypothesis is preferred as, in addition to its being more parsimonious, there is some evidence to suggest that the *L.* (*Sauroleishmania*) clade is evolving faster (see text). The dashed line shows the 20°C isotherm for July in the northern hemisphere. Sandflies require approximately 50 consecutive days with a temperature above 20°C in order to breed and the 20°C isotherm at approximately 45°N marks the northern limit of sandfly distribution. The floral province of Beringia is situated between 60°N and 70°N and is the most probable route for the migration of *Leishmania* from the Neotropics to the Old World. Since the Old World *Leishmania* consist of one or at most two clades the passage across Beringia must have been a rare and difficult event. The last time that Beringia would have been warm enough for phlebotomine sandflies was probably in the mid-Miocene (26–13 mybp) (Wolfe, 1994a, b), which is probably the latest date at which *Leishmania* could have migrated to the Old World. The earliest date is set by the arrival of caviomorph rodents in the Neotropics at the Eocene/Oligocene boundary, about 37 mybp.

parasites of edentates. The secondary splits both within the genus *Leishmania s.s.* and within the sister clade, which includes *L. hertigi* of porcupines and *Endotrypanum* of sloths, would then have occurred between parasites of edentates and those of caviomorph rodents. These secondary splits cannot have occurred until the late Eocene (35 mybp), when the caviomorph rodents arrived in the Neotropics.

The isoenzyme and DNA data also support, or are consistent with, a Neotropical origin of the *Leishmania/Endotrypanum* clade. This clade may have made the switch from monogenetic parasites of phlebotomine sandflies to digenetic parasites of mammals during the late Cretaceous or early Cenozoic (95–37 mybp), after the Neotropics had become isolated from Africa and North America. Ancestral *L. (Leishmania)* spp. must have migrated to the Old World by the mid-Miocene at the latest, before the Bering region became too cold for phlebotomine sandflies (Wolfe, 1994a, b; Figure 8). The lizard *Leishmania* may have developed as a consequence of the intense challenge of infection to lizards resulting from their sharing burrow systems with infected rodents and phlebotomine sandflies (Noyes *et al.*, 1997). Accordingly, it has been predicted that the sandflies of the Nearctic and Palaearctic share a recent common ancestor (Noyes, 1998b). Although no such common relationship is reflected in the majority of classifications of the Phlebotominae, the higher classification of this subfamily is unstable and controversial (Lane, 1993) and it has been asserted that the existing genera were established on the basis of geography and not morphology (Ashford, 1991). Recent morphological and isoenzyme studies are not consistent, but have independently shown closer relationships between the New and Old World phlebotomine vector subgenera than with sympatric non-vector congeneric subgenera (Galati, 1995; Dujardin, J.P. *et al.*, 1999b). A major DNA-based study is required to resolve the phylogeny of the phlebotomine sandflies with confidence and to provide an essential test for the hypothesis of a New World origin of *Leishmania*.

5.5. Other DNA Sequences

Conclusions relating to *L. equatoriensis/L. colombiensis* and *Endotrypanum* based on RFLP analysis of the ssu rRNA (Section 5.3, p. 36) and RNA/DNA polymerase sequences (Section 5.4, p. 38) are confirmed by the work of Cupolillo *et al.* (1998), who used short (83 bp) sequences from the conserved region of kinetoplast DNA minicircles and isoenzyme data to confirm the close relationship between *L. equatoriensis/L. colombiensis* and *Endotrypanum* species (Figure 6).

Although the RNA and DNA polymerase gene phylogenies are useful for classifying the *Leishmania* species complexes, these genes are not evolving

fast enough to resolve the relationships within the species complexes. The only DNA sequence that has so far been shown to resolve *Leishmania* species is a repetitive element originally identified in *L. infantum*. This sequence has been used to classify the majority of the Old World mammalian *Leishmania* species (Piarroux *et al.*, 1995). The resulting phylogeny agrees in many respects with the isoenzyme phylogeny of the same strains derived by Rioux *et al.* (1990). However, the phylogeny based on repetitive DNA sequence classifies *L. aethiopica* as a sister clade of *L. tropica*, as do the DNA and RNA polymerase gene phylogenies, whilst the isoenzyme phylogeny classifies *L. aethiopica* as a sister clade of *L. major*. The tree based on the repeat sequence data also indicates that both the *L. tropica* and *L. major* clades are polyphyletic, whereas the same strains appear to form monophyletic groups when analysed by isoenzymes. It should perhaps be borne in mind, however, that the evolutionary significance of non-coding sequences is still poorly understood, since the constraints on mutation of these sequences are quite different from those governing the evolution of the organism as a whole.

5.6. Outlook

There is now a substantial body of molecular evidence for a Neotropical origin of the modern genera *Leishmania* and *Endotrypanum*. DNA and RNA polymerase gene phylogenies have proved invaluable for the classification of the major *Leishmania* species complexes; however, some parts of even these phylogenies are still not strongly supported and it will be necessary to include additional taxa and possibly to employ additional genes before the true species phylogeny of *Leishmania* can be determined with confidence. Moreover, while a number of techniques has been shown to be suitable for identifying and classifying *Leishmania* strains within species complexes, these all depend on the availability of a bank of reference strains. The best developed of these methods is still MLIE, which is supported by a substantial zymodeme database and collections of cryopreserved material (e.g. Rioux *et al.*, 1986). The development of new methods should be based on characters that do not require the support of a large bank of reference strains. Either DNA sequencing or DNA chips could be the method of choice in future and the challenge will be to identify genes or polymorphisms that are as useful as isoenzymes for characterizing *Leishmania* strains. Use of the repeat sequence identified by Piarroux *et al.* (1995) has shown what can be done, but further sequences should be tested before one is selected to answer the needs of the *Leishmania* research community in the decades ahead.

6. CONCLUSIONS

The first molecular characterization studies of Trypanosomatidae were not conducted with evolutionary aims, but primarily for identification of morphologically identical parasite strains. Over the years a range of so-called intrinsic characters has been used to analyse trypanosomatid genotypes, at first indirectly, e.g. isoenzymes, and then directly, e.g. RFLPs in kinetoplast and nuclear DNA. With the advent of automated DNA sequence analysis, it is now feasible to investigate variation at the ultimate level, that of the complete gene sequence, and this in turn has opened the door to reliable phylogenetic reconstruction. The application of this approach to trypanosomatid evolution has already challenged some long-cherished beliefs, such as the 'invertebrate-first' model of trypanosomatid evolution, and there will doubtless be more surprises to come. Future studies of trypanosomatid evolution will also benefit from developments in parallel subject areas, such as vector phylogeny and the biology of parasite–vector interactions. In particular, the complexity of some trypanosomatid developmental cycles in their vectors should be a good indicator of the age of these associations.

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presented interesting results suggesting that the two *T. cruzi* lineages diverged between 88 and 37 million years before present (mybp), after which they underwent separate evolutionary histories in North and South America, related to the particular mammalian faunas of the two regions (Briones *et al.*, 1999). Michael Gaunt and Michael Miles (London School of Hygiene and Tropical Medicine) added that the often largely overlooked edentates, especially armadillos, may have a significant role to play in unravelling the evolutionary history of the *T. cruzi* lineages.

Using an estimate of divergence based on the molecular clock presented by Escalante and Ayala (1995) [see p. 44], Briones *et al.* (1999) also suggested that *T. rangeli* diverged from *T. cruzi* some 475 mybp, several million years before the evolution of early insects (~380 mybp) and the first land vertebrates (~360 mybp). Such a divergence date is at severe odds with the Late Cretaceous/Tertiary mammal-associated divergence hypothesis presented by Chris Schofield (ECLAT/London School of Hygiene and Tropical Medicine) and, in the light of discussions relating to biogeographical associations presented by C. Barry Cox (King's College, University of London, UK) at the workshop, we suggest that such a time estimate highlights further the need for independent calibration of molecular clocks, particularly when attempting to extrapolate rates over long periods of geological time. [The clock-based divergence estimates of Briones *et al.* (1999) are also vastly different from the clade composition/biogeography-based divergence estimates presented in this review – see Figure 4, p.14.]

Alternative hypotheses on the evolution of *Leishmania* – Neotropical, Palaearctic, African – were presented at the workshop by Harry Noyes (University of Liverpool, UK), Sara Kerr (University of the Incarnate Word, Texas, USA) and Hooman Momen (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil) respectively, reflecting differences in the research focus of the contributors (parasite versus host) and highlighting uncertainties still surrounding the evolution of these parasites (Noyes, 1998b [see p. 49]; Kerr, 2000). Based on the observation that rodents are the principal hosts of *Leishmania* in both the Nearctic and the Palaearctic, Sara Kerr proposed that the mammalian *Leishmania* evolved from lizard *L. (Sauroleishmania)* in the Palaearctic and that they then migrated to the Nearctic in the Oligocene and to the Neotropics after the Isthmus of Panama had formed in the Pliocene (3–5 mybp). A number of concerns relating to this hypothesis were raised by Harry Noyes, principally that a Palaearctic origin is inconsistent with published molecular phylogenies of *Leishmania* (Sections 5.3, p. 36 and 5.4, p. 38; Croan *et al.*, 1997 [see p. 43]; Noyes *et al.*, 1997 [see p. 49]). Kerr (2000) proposed that this inconsistency is a consequence of a rapid rate of evolution in the Neotropical *Leishmania*; however no evidence was presented to show that an increased rate of evolution could convincingly reconcile the phylogeny required by a Palaearctic origin with published phylogenies. Given that *Leishmania* can infect at least eight

orders of mammals, it appears somewhat speculative to propose a hypothesis for an origin in the Palaearctic based on an association with just one of these host orders.

The contradictory hypotheses of Kerr and Noyes are a consequence of incongruence between extrinsic host data and intrinsic parasite data, respectively. However, an evolutionary interpretation of the intrinsic parasite data requires fewer assumptions than does the interpretation of extrinsic host data and, thus, it seems probable that relationships defined on the basis of intrinsic data will be more robust. Accordingly, while data from host associations and distribution may be important for aiding the interpretation of intrinsic parasite data, and sometimes for detecting gross problems with molecular phylogenies, extrinsic data are probably not the most appropriate basis for constructing primary evolutionary hypotheses.

Also within the *Leishmania* genus of trypanosomatids, Elisa Cupolillo (Instituto Oswaldo Cruz) presented a new approach to resolving the status of *Leishmania* isolates/species which consistently group with *Endotrypanum* species using biochemical and molecular markers (see Section 5, p. 31) (Cupolillo *et al.*, 2000). All data indicated the presence of two major phylogenetic lineages within *Leishmania* that are clearly divergent; the lineage represented by *L. (Leishmania)* and *L. (Viannia)* was named Euleishmania and the other, containing *L. hertigi, L. deanei, L. herreri, L. colombiensis* and *L. equatorensis*, together with all species and strains currently classified within the genus *Endotrypanum*, was assigned the name Paraleishmania. These lineage names have no taxonomic status and are analogous to the terms Salivaria and Stercoraria for *Trypanosoma*. The generic name *Endotrypanum* is to be reserved for the real intraerythrocytic parasites of sloths when their life cycle is fully established.

Many of the papers presented at the trypanosomatid evolution workshop are available in the *Memórias do Instituto Oswaldo Cruz* (2000), volume 95, part 4.

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Transovarial Transmission in the Microsporidia

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ABSTRACT

The microsporidia are an ancient and diverse group of protists which have many unusual characteristics. These include prokaryotic-like 70s ribosomes, enclosed nuclear division, a lack of mitochondria and complex life cycles

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which frequently involve vertical transmission. This use of vertical transmission is unparalleled by other protists and is seen only among bacterial endosymbionts and sex ratio distorters and in host cell organelles. Transovarially transmitted microsporidia can have unusual and profound effects on host population sex ratios. We here consider the mechanisms of transovarial transmission and its implications for parasite evolution. We review parasite/host relationships and the evolution of virulence under transovarial transmission and consider the implications of these parasites for host ecology and evolution.

1. INTRODUCTION

1.1. Vertical Transmission

Parasite transmission occurs through a number of different routes which fall into two categories: horizontal and vertical transmission. Horizontal parasite transmission can occur between related or unrelated hosts of the same or different generations with parasites transmitted *per os*, venereally and by direct invasion through the host epithelium. Vertically transmitted parasites are passed from generation to generation of hosts and transmission takes place within a host lineage. Vertical transmission has been reported for a diverse range of parasites including viruses, bacteria, protists and helminths.

There are a number of routes of vertical transmission. Amongst mammal hosts, transmission may occur via the placenta of the infected female host. Parasites that are transmitted in this way are often also passed on to the offspring after birth via the mammary glands. Transplacental transmission has been reported for a number of parasites including HIV and malaria in humans (Redd *et al.*, 1996; Saglio *et al.*, 1996), *Toxocara canis* in dogs (Kassai, 1995), *Toxoplasma gondii* in various mammals including humans (Remington and Desmonts, 1983; Blewett and Watson, 1984) and numerous helminths in humans and other mammals (Eberhard *et al.*, 1993; Shoop, 1994). There are few data on transplacental transmission amongst the microsporidia, although this route has been demonstrated for *Encephalitozoon cuniculi* in rabbits and in mice (Innes *et al.*, 1962; Hunt *et al.*, 1972). Transmammary transmission has been recorded for helminths (Shoop, 1994) and HIV (Saglio *et al.*, 1996).

Transovarial transmission is the most important vertical route amongst parasites of invertebrates and is used by a number of intracellular parasites including viruses, bacteria and protists (Dunn *et al.*, 1995). These parasites are located in the cytoplasm of the host's ova at reproduction and are transmitted via the gametes to the offspring (cytoplasmic inheritance). For example, transovarial transmission has been reported for the Ross River virus infecting the mosquito *Aedes vigilax* (Vale *et al.*, 1992), for a diverse range of bacteria

infecting insects and crustaceans (Werren and O'Neill, 1997), for a haplosporidian-like parasite of crustaceans (Ginsburger-Vogel and Desportes, 1979) and for a number of microsporidia infecting insects and crustaceans (e.g. Kellen *et al.*, 1965; Dunn *et al.*, 1995). For most of these parasites, as male gametes are extremely small and rarely contribute to the cytoplasm inherited by the zygote, transmission is uniparental (maternal). Only female hosts transmit the infection and a parasite in a male host is at an evolutionary dead end unless it can attain transmission by some other, horizontal route. Transovarial transmission of parasites has parallels with the maternal inheritance of cell organelles such as mitochondria, chloroplasts and plastids.

In the microsporidia, horizontal transmission is the most common mode of transmission. Spores are usually ingested by the host where they infect the gut epithelium and may spread to other host tissues (Canning and Lom, 1986). However, many microsporidia are also transovarially transmitted to new hosts. For some microsporidia, transovarial transmission is a minor route supplementary to the main horizontal route. However, for a large subset of microsporidia of arthropods, transovarial transmission is crucial for parasite maintenance in the host population and transmission strategies include alternating horizontal and transovarial routes as well as sole transovarial transmission (Table 1, e.g. Lucarotti and Andreadis, 1995; Koella and Agnew, 1997; Terry *et al.*, 1998).

1.2. Diversity, Disease and Distribution

Microsporidia are known to infect a range of host species from protists to humans, but approximately half of all microsporidian genera have been described from insects and it was in this sphere that their economic importance was first noted. *Nosema bombycis,* Naegeli 1857 was the first described microsporidian species. This parasite was demonstrated to cause pebrine, a disease that heavily affected commercial colonies of the silkworm *Bombyx mori*. *N. bombycis* caused severe gut pathology and was primarily transmitted horizontally by ingestion of spores but, interestingly, vertical (transovarial) transmission was also found to occur. A second species of *Nosema, N. apis*, causes severe pathology in honeybees. In insects, disease pathology is proportional to the number of spores generated and the most common infection sites are the gut or the fat body (Canning, 1982; Larsson, 1988). Infection has significant effects on the growth and survival of insects and for this reason microsporidia have been proposed as biological control agents for pests and disease vectors (Canning, 1982). However, this use is limited as massive doses of spores are required to influence the host population and the host specificity of some parasites may restrict application. Although horizontal transmission is most frequently reported, many microsporidian species infecting insects make use of transovarial transmission during their life cycle (Andreadis, 1985a; Canning *et al.*, 1985; Becnel *et al.*, 1989; Andreadis, 1990).

Table 1 Examples of transovarial transmission amongst the microsporidia. Transovarial transmission has been reported for a range of microsporidian genera in insect and crustacean hosts. In the majority of systems, both vertical and horizontal transmission routes are used. Most life cycles are direct, although some involve an alternate host. The use of transovarial transmission as the sole transmission route has been reported for a small subset of microsporidia.

Parasite	Host	Transmission	Authors
Amblyosporidae			
Amblyospora campbelli	*Culiseta incidens*	Transovarial and horizontal	Dickson and Barr, 1990
Amblyospora indicola	*Culex sitiens* and *Apocyclops* sp.	Transovarial and horizontal (direct and indirect)	Sweeney *et al.*, 1990
Parathelohania anophelis	*Anopheles quadrimaculatus* and *Microcyclops varicans*	Transovarial and horizontal (direct and indirect)	Avery and Undeen, 1990
Burenellidae			
Pilosporella chapmani	*Aedes triseriatus*	Transovarial and horizontal	Becnel *et al.*, 1986
Caudosporidae			
Octosporea effeminans	*Gammarus duebeni*	Transovarial only	Bulnheim and Vavra, 1968
Culicosporidae			
Culicospora magna	*Culex restuans*	Transovarial and horizontal	Becnel *et al.*, 1987
Edhazardia aedis	*Aedes aegypti*	Transovarial and horizontal	Becnel *et al.*, 1989
Nosematidae			
Nosema bombycis	*Bombyx mori*	Transovarial and horizontal	Han and Watanabe, 1988
Nosema empoascae	*Empoasca fabae*	Transovarial only	Ni *et al.*, 1997
Nosema fumiferanae	*Choristoneura fumiferana*	Transovarial and horizontal	Thomson, 1958
Nosema granulosis	*Gammarus duebeni*	Transovarial only	Terry *et al.*, 1997

Table 1 cont.

Parasite	Host	Transmission	Authors
Pleistophoridae			
Pleistophora oncoperae	*Oncopera alboguttata*	Transovarial and horizontal	Milner and Lutton, 1980
Thelohaniidae			
Thelohania hereditaria	*Gammarus duebeni*	Transovarial only	Bulnheim, 1971
Thelohania solenopsae	*Solenopsis richteri*	Transovarial	Briano *et al.*, 1996
Unikaryonidae			
Orthosomella operophterae	*Operophtera brumata*	Transovarial and horizontal	Canning *et al.*, 1985

Microsporidia also cause disease in fish, particularly in commercial hatcheries (Hauck, 1984; Kent *et al.*, 1989) and aquaria (Lom *et al.*, 1995). Disease pathogenesis varies, with some genera such as *Glugea* and *Loma* causing the formation of xenomas. The xenoma is formed as a result of hypertrophy of the infected cell. The resulting structure is up to several millimetres in diameter, the internal cytoplasm is filled with developing parasites and the membrane specialized for nutrient uptake (Canning and Lom, 1986). The mature xenoma breaks down to release spores and the ensuing tissue damage leads to granuloma formation (Dykova *et al.*, 1980; Ralphs and Matthews, 1986). Other genera, such as *Pleistophora*, do not induce host cell hypertrophy but cause massive disruption in muscle and other tissues (Pulsford and Matthews, 1991). Some *Pleistophora* species infect oocytes but as they are highly pathogenic it is not clear whether this constitutes a vertical transmission route or if the oocytes are destroyed (Summerfelt and Warner, 1970).

Until recently, microsporidian parasites infecting mammals were thought to be almost exclusively from the genus *Encephalitozoon*. These parasites have been reported from a wide range of mammals (Bornay-Llinares *et al.*, 1998). In specific surveys parasite induced lesions are often found, particularly in brain and kidney, but clinical disease is rarely seen (Gannon, 1980). *Encephalitozoon* is thought to be a zoonotic disease that is transmitted either by ingestion of spores (Desplazes *et al.*, 1996) or by vertical, transplacental, transmission (Hunt *et al.*, 1972; Owen and Gannon, 1980). Prior to the advent

of AIDS, human microsporidiosis was very rare, however in AIDS patients the disease has been much more frequently reported and the number of genera giving rise to disease has increased (Shadduck, 1989; Didier *et al.*, 1998). *Encephalitozoon* species are the most common giving rise to disseminated infection and gut pathology (Molina *et al.*, 1993; Weber *et al.*, 1993), ocular infection also occurs (Friedberg *et al.*, 1993). The disease in AIDS patients is often referred to as opportunistic, but it is difficult to imagine that AIDS patients have any greater opportunities for infection and seems likely that the same parasites result in sub-clinical infection in immunocompetent hosts.

In summary, microsporidia are a diverse group which infect a wide range of hosts. Some species are highly pathogenic, the damage usually being related to the production and release of spores for horizontal transmission. Vertical

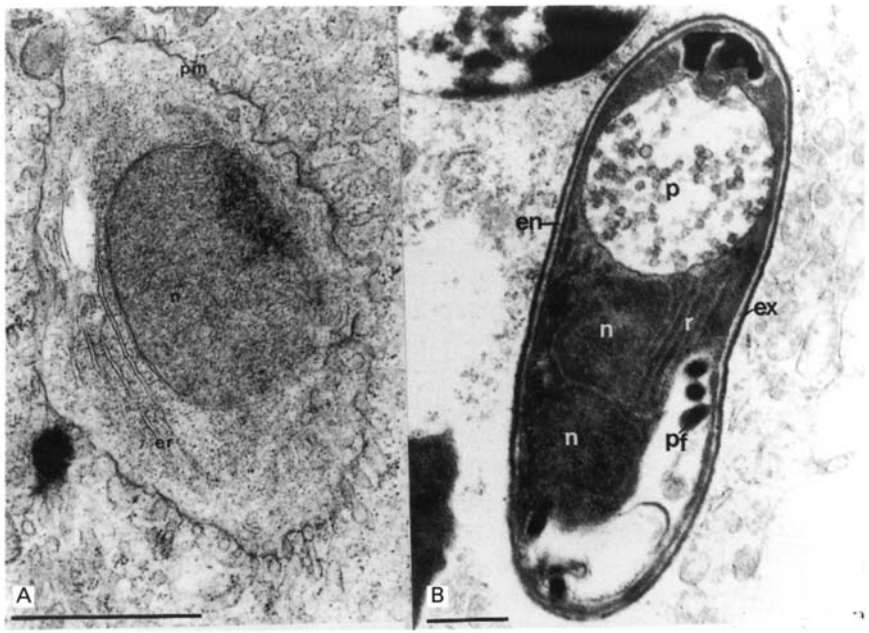


Figure 1A Electron micrograph showing a meront of *Nosema granulosis* infecting a *Gammarus duebeni* embryo. The meront has an undulating plasma membrane (pm) with finger-like projections. The cytoplasm of the meront contains endoplasmic reticulum (er) and the nucleus (n). Scale bar = 1 μm (Terry *et al.*, 1997).

Figure 1B Electron micrograph showing a spore of *Nosema granulosis* infecting the ovarian tissue of a *Gammarus duebeni* host. The spore has exospore (ex) and endospore walls (en), nucleus (n) in diplokaryotic arrangement, polyribosomes (r), a polar filament (pf) with 3 coils and a polaroplast (p) with unusual globular contents. Scale bar = 500 nm (Terry *et al.*, 1997).

(transovarial or transplacental) transmission is also seen in the life cycle of a number of different microsporidian genera. The importance of this form of transmission is almost certainly underestimated as it is rarely associated with host pathology and may be overlooked in many systems.

1.3. Cellular Structure and Function

Microsporidia are true intracellular parasites that reside directly in the host cell cytoplasm and are unable to grow or divide outside the host cell. There are two basic morphological forms, the meront or proliferative stage and the spore or transmission stage.

The meront stage has a very simple cellular structure (Figure 1A). It has a nucleus that may be isolated or paired (diplokaryotic) and is enclosed within a nuclear envelope which remains intact throughout mitotic division (Vavra, 1976a; Raikov, 1982). In diplokarya, the nuclei divide synchronously but remain separated from each other (Vavra, 1976b). Meronts have abundant rough endoplasmic reticulum, which is studded with 70s ribosomes (Ishihara and Hayashi, 1968) and, although there is no conventional Golgi apparatus, a specialized area of dense membrane bound vesicles has been noted (Vavra, 1976a). The cytoplasm is virtually devoid of organelles with no mitochondria, peroxisomes, hydrogenosomes, glycosomes or nutrient storage granules. The meront plasma membrane is in direct contact with the host cell cytoplasm and is often folded and undulating, presumably to increase the area available for nutrient uptake. There are signs that the parasite is also able to exploit host organelles as a source of nutrients and contact has been noted both between the meront surface and host endoplasmic reticulum and between the meront and the mitochondria (Sokolova *et al.*, 1988; Cali and Owen, 1990).

In contrast to the meront, spore stages have a high level of structural organization. The spores possess two adaptations for their specialized role in parasite transmission, a complex extrusion apparatus and an outer protective coat (Figure 1B). The outer coat of the spore enables survival in the extracellular environment. It consists of two layers, the exospore and the endospore. The exospore is highly variable in structure and thickness, it often appears as concentric layers of differing electron density and is sometimes decorated with external spikes, or tubular appendages (Rauch and Grunewald, 1980; Bigliardi *et al.*, 1986). The endospore is an electron lucent, chitinous layer which lies immediately above the plasma membrane (Vavra, 1976a; Vavra and Chalupsky, 1982). The extrusion apparatus enables the parasite to infect new host cells. It consists of the polar filament, anchoring disc, polaroplast and posterior vacuole. The polar filament is a coiled tube that arises from a membrane bound polar sac at the anterior of the spore. Within this polar sac the filament forms a carbohydrate rich attachment plaque, the anchoring disc, which is

associated with the plasma membrane at a point where the endospore wall is thin (Lillie, 1965; Vavra *et al.,* 1986). The length and gross morphology of the filament varies but there is usually a straight section with an electron dense core (the manubrial filament) followed by a series of coils. The filament itself has a thick wall composed of protein and carbohydrate and appears to be enclosed within a unit membrane that may originate from the polaroplast (Vavra, 1976a). The polar sac and manubrial filament are surrounded by the polaroplast, an extensive membranous organelle which usually takes the form of closely stacked lamellae or tubules (Sprague *et al.,* 1968; Canning and Nicholas, 1980). The central portion of the spore contains the nucleus and the cytoplasm which is often packed with polyribosomes (Cali and Owen, 1988). Finally a membrane bound vacuole is often seen in the posterior section of the spore (Lom and Corliss, 1967; Canning and Lom, 1986).

Spore germination is triggered by external stimuli including pH and ion concentrations (Undeen and Epsky, 1990). In response to these stimuli, there is a net influx of water that results in changes in the internal structure. The polaroplast lamellae unravel and the posterior vacuole expands displacing the cytoplasm, the polar filament ruptures through the spore wall at the site of the anchoring disc and is everted with such force that it is able to puncture the host cell membrane (Lom and Vavra, 1963). The parasite sporoplasm (nucleus and cytoplasm) then passes through the polar filament directly into the host cell cytoplasm (Lom, 1972). Eversion of the filament is believed to be due to 'swelling' of the spore caused by the influx of water, but this should not be regarded as a simple physical process. Eversion of the filament alone requires localized disruption of the plasma membrane and reorganization of the filament structure including the polymerization of polar filament proteins into the extending structure (Weidner *et al.,* 1995). Undeen (1990) suggests that the key event is the degradation of trehalose to glucose and concomitant increase in internal osmotic pressure. There is also evidence that germination is Ca^{++} regulated (Weidner and Byrd, 1982; Frixione *et al.,* 1994; Weidner *et al.,* 1995) and may well involve signalling pathways (Weidner and Halonen, 1993) which would trigger the complex structural and metabolic changes.

1.4. Microsporidian Phylogeny and the Evolutionary Origins of Transovarial Transmission

Although transovarial transmission has been reported in several genera its distribution among the microsporidia has never been systematically studied. It is interesting to speculate on the evolutionary origins of this transmission route within the phylum and whether any specific adaptations are required for its use. To address this question we must first consider the phylogeny of microsporidia.

The phylum Microspora are a diverse group and their taxonomy undergoes frequent revisions (Sprague and Vavra, 1977; Larsson, 1986; Sprague *et al.*, 1992). Classification is largely based on spore structure and the developmental cycle of the parasite. Ecological data are given little weight and factors such as host range or transmission mechanism are not taken into account. The gross morphology of microsporidian spores is variable with a size range of 1–40 μm and shapes can range from ovoid to filiform (Becnel *et al.*, 1989; Olsen *et al.*, 1994). The exospore wall varies in structure and thickness and may have elaborate external appendages. The polar filament provides several criteria, it may be of an even thickness (isofilar) or tapering (anisofilar) and variation in its length results in differences in the number of coils and in the manner in which they are packed into the spore (Larsson, 1986).

The developmental cycle of the parasite provides further taxonomic criteria. Sporogenesis, the transition from meront to spore stage, is a highly variable process. The first event is usually a thickening of the meront plasma membrane to form a sporont but thereafter there is considerable diversity among microsporidian genera (Sprague *et al.*, 1992; Cali and Takvorian, 1999). In the genus *Nosema*, for example, the thickened membrane forms the exospore wall. Parasite division leads to the formation of paired sporoblasts that differentiate, forming spores that lie directly in the host cell cytoplasm. In *Pleistophora*, the proliferative phase leads to the formation of a multinucleate plasmodium which secretes a thickened coat. The coat then separates from the parasite plasma membrane forming a structure known as a sporophorous vesicle that segregates the developing parasites from the host cell cytoplasm. Cell division continues within this envelope to produce many uninucleate spores (Canning and Hazard, 1982). In other genera e.g. *Septata* (Cali *et al.*, 1993), spores are grouped within a membrane of host origin. The mono- or diplokaryotic nature of the parasite and the way in which gametes are formed, by meiosis or dissociation, are also important taxonomic criteria (Sprague *et al.*, 1992).

The classification of microsporidia using these morphological traits results in a complex and somewhat confusing taxonomy. Sprague *et al.* (1992), for example, recognized 143 genera in the phylum, many of which contain a single species described in a single host. Some problems arise due to differences in the quality of information generated by light and electron microscopy. A more serious concern, however, is that life cycles are complex and species descriptions do not always contain full information. For example, *Vairimorpha* has two sporulation cycles one of which occurs directly within the cytoplasm in a similar way to that of parasites of the genus *Nosema*. However, it also has a second sporulation cycle which occurs within a sporophorous vesicle, producing groups of eight spores (Mitchell and Cali, 1993). This hybrid life cycle casts some doubt on the validity of the sporophorous vesicle as a taxonomic marker. It should be remembered that microsporidia may be polysporous and can have several different sporulation sequences during their life cycle

(Johnson *et al.*, 1997). Problems that arise with classification may in part be due to incomplete taxonomic descriptions that fail to take account of morphologically distinct spores in the life cycle. This is illustrated by the recent discovery of 'early' or autoinfection spores from a number of genera (Sagers *et al.*, 1996; Johnson *et al.*, 1997).

More recently the relationships between microsporidia have been analysed using molecular techniques. This approach should enable some of the controversies over developmental cycles and alternate transmission mechanisms to be resolved. Comparisons currently rest on sequence data generated from rRNA genes, in particular the small subunit 16s rRNA (Vossbrinck *et al.*, 1993; Baker *et al.*, 1995; Pieniazek *et al.*, 1996). This analysis is somewhat biased by the inclusion of a disproportionate number of species associated with severe pathology in AIDS patients and insects but nevertheless some interesting results are being generated. For example, the type species of the *Vairimorpha* (*V. necatrix*) was closely related to a clade of the genus *Nosema* which is defined as having spores directly in the cytoplasm (Baker *et al.*, 1994). Interestingly the transovarially transmitted microsporidium, *Nosema granulosis*, which has only a single spore type, is found in the other *Nosema* clade along with the type species *N. bombycis* (Terry *et al.*, 1999b).

Integration of morphological and molecular data should, over the next few years, bring new insights into the phylogeny of the Microspora. It might also be instructive to incorporate ecological factors such as host range and transmission route in this analysis. It is clear that transovarial transmission occurs widely across the phylum (Summerfelt and Warner, 1970; Hunt *et al.*, 1972; Andreadis, 1985a; Terry *et al.*, 1998) and it is interesting to question whether it constitutes an ancestral transmission route or results from several separate introductions. In order to address these questions we need to re-examine parasite transmission in many genera as the potential for transovarial transmission has never been directly assessed.

1.5. Parasite Adaptations for Transovarial Transmission

It is not possible to describe a typical life cycle for microsporidian parasites and it is instructive to compare a few different species in order to evaluate the role of transovarial transmission. The simplest life cycles involve direct transmission within a single host species. This is illustrated by *Nosema apis,* an economically important species which infects honeybees. Spores are ingested and germinate in the midgut infecting epithelial cells. Meronts divide in these cells but after 48 hours some differentiate to form 'early' spores which germinate spontaneously to infect adjacent epithelial cells. Other meronts divide and differentiate to form large numbers of thick walled spores which destroy the host cell and are passed out in the faeces to transmit the infection (de Graaf *et*

al., 1994). In this system horizontal transmission is clearly the major route but the potential for transovarial transmission has not yet been assessed. In other species transovarial transmission forms an obligate step in the life cycle (Figure 2). For example, *Ambylospora connecticus,* a species that infects mosquitoes, produces three spore types in a cycle that involves transovarial transmission from the adult mosquito host to larvae and subsequent horizontal transmission to adults through a copepod intermediate host (Andreadis, 1985a, b). Finally, there are species which are exclusively transovarially transmitted

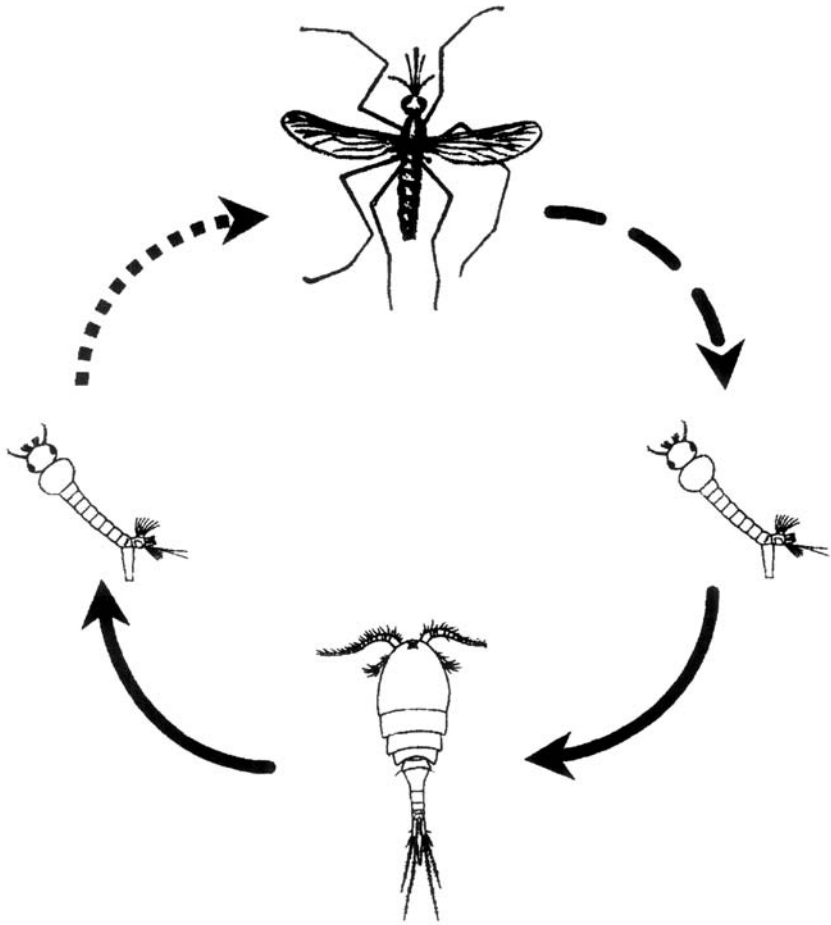


Figure 2 Diagrammatic representation of the life cycle of *Amblyospora connecticus.* This microsporidian has alternating horizontal and vertical transmission and has an indirect life cycle involving a mosquito and a copepod intermediate host. Solid line represents horizontal transmission, dashed line indicates vertical transmission, dotted line indicates transtadial transmission.

such as *Nosema granulosis* (Terry *et al.*, 1999b). In this parasite, there is only a single spore type which is found in the gonad of the female host and infects oocytes. In infected juvenile animals only a few meront stages are seen and these migrate to the gonadal tissue where sporogenesis takes place (Terry *et al.*, 1997, 1999a).

In comparing these life cycles it is evident that transovarial transmission is employed in very different ways by different species of microsporidia. The only common features that emerge are, that transovarial transmission is rarely associated with host pathology and that it appears to be mediated by a specific spore type (Iwano and Ishihara, 1991; Terry *et al.*, 1997). The characteristics of this transovarially transmitted spore closely resemble those of the 'early' or auto-infection spores (Iwano and Ishihara, 1991; Sagers *et al.*, 1996; Johnson *et al.*, 1997). Their features include a short polar filament, a thin endospore wall and sometimes an unusual polaroplast, all of which may represent adaptations to intracellular germination and infection of locally adjacent cells. In recent studies of early infection this spore type has been reported in a number of genera and it may prove to be of widespread or even ubiquitous importance in dissemination of disease within the host. These findings leave us with an interesting question. Were the 'early' spore and transovarial transmission ancestral in the microsporidia or is *N. granulosis* a degenerate *Nosema* parasite which has lost part of its life cycle?

1.6. The Parasite as an Endosymbiont

In reviewing the cell biology of microsporidia it is evident that the parasite is truly intracellular and that, due to the unusual infection mechanism, the plasma membrane is only ever in contact with host cytoplasm. This may well have contributed to one of the most striking features of microsporidia, i.e. their association with host endoplasmic reticulum and mitochondria (Cali and Owen, 1990; Canning and Hollister, 1992; de Graaf *et al.*, 1994; Terry *et al.*, 1997). The interaction with host mitochondria is particularly interesting as microsporidia lack mitochondria themselves and the question arises as to whether they originally had mitochondria and lost them in adopting an intracellular lifestyle. Some evidence in favour of secondary loss is that genes encoding homologues of HSP70, a mitochondrial import protein, have been characterized from two microsporidian species (Germot *et al.*, 1997; Hirt *et al.*, 1997). If the parasite were indeed secondarily amitochondrial it is likely that other mitochondria associated genes will be found. Alternatively, HSP70 may have a secondary function unconnected to mitochondrial protein import. Whatever the outcome of these studies it is clear that the association between the parasite and host mitochondria is very strong and indicative of a functional relationship. In *Nosema granulosis* EM studies have shown that mitochondria are flattened along the parasite plasma

membrane and the two membranes are closely juxtaposed in a structure which resembles a gap junction (Figure 3A,B). These morphological data suggest metabolic interaction between the parasite and organelle and this is supported by the observation that mitochondria attached to the parasite were larger than those free in the cytoplasm (Terry *et al.*, 1999a). Although there is no direct evidence of molecular transfer it has been shown that isolated parasites are able to utilize exogenous sources of ATP (Weidner *et al.*, 1999).

Again in *N. granulosis* there is evidence that the parasite is even further integrated into the cellular apparatus of the host. Studies of parasite distribution during mitotic division of infected embryos revealed interaction with the host cell cytoskeleton (Terry *et al.*, 1998). In interphase cells, meronts have a perinuclear distribution but during division they are drawn towards the spindle poles and segregate along the axis of division (Figure 4A–D). Electron microscopy reveals that the parasite has both direct, and indirect (via mitochondria) links with host cell microtubules (Terry *et al.*, 1998). The distribution of parasites among host cells during embryogenesis is not even (Dunn *et al.*, 1998) and this implies that the parasite and its associated mitochondria may target specific cell lineages, such as putative gonadal tissue. *N. granulosis* is a transovarially transmitted microsporidium which feminizes its host, converting males into females. In this system, it is clear that the physical association between the parasite and the mitochondria will bias mitochondrial selection. It is tempting to speculate that transovarial transmission was the ancestral route

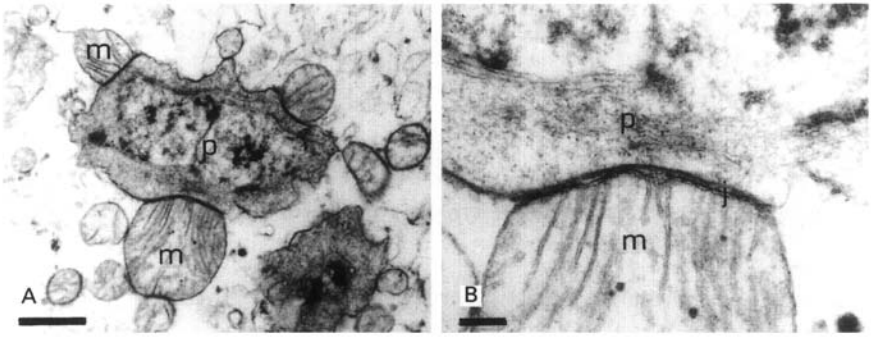


Figure 3A Electron micrograph showing the relationship between parasite and host mitochondria for *Nosema granulosis* in a host (*Gammarus duebeni*) embryonic cell at metaphase. Parasite (p) meronts can be seen in close association with 3 host mitochondria (m). Scale bar = 1 μm (Terry *et al.*, 1999a).

Figure 3B EM showing the junction (j) between parasite meront (p) and a host mitochondrion (m) in a *Gammarus duebeni* embryo infected with *Nosema granulosis*. The parasite plasma membrane and the outer membrane of the mitochondrion are flattened together to form a junction. Scale bar = 200 nm (Terry *et al.*, 1999a).

in microsporidia and that co-inheritance of host mitochondrial and parasite genomes led to the loss of parasite mitochondria.

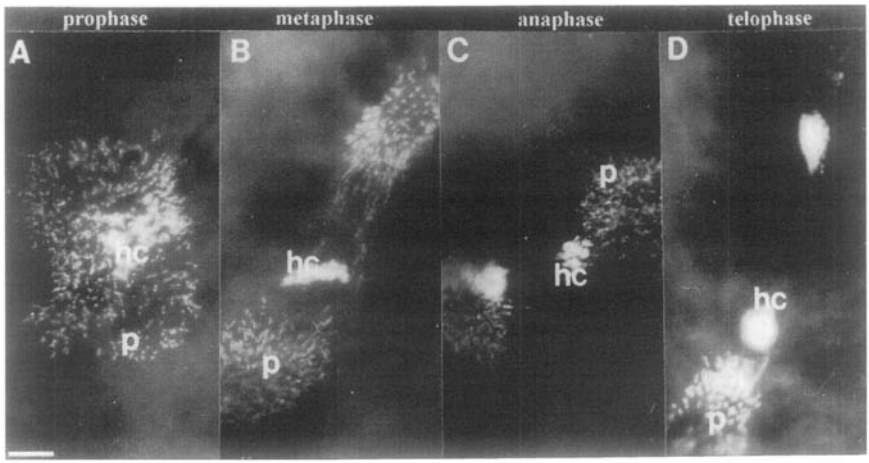


Figure 4A–D The distribution of *Nosema granulosis* meronts during mitosis of a host (*Gammarus duebeni*) embryonic cell. Host (hc) and parasite (p) nuclei are stained with DAPI, a DNA fluorescent dye. *A. Prophase.* The parasites are uniformly distributed around the condensing host chromosomes. *B. Metaphase.* The host chromosomes are aligned on the spindle plate and the parasites are located in two distinct clusters at the opposite poles of the dividing nucleus. *C. Anaphase.* The host chromosomes have moved towards the spindle poles while the parasites remain in this area. *D. Telophase.* The parasites are associated with the two re-organizing daughter host nuclei. Scale bars = 0.01 mm (Terry *et al.*, 1999a).

2. MECHANISMS OF TRANSOVARIAL TRANSMISSION

Understanding of the mechanisms involved in parasite movement within the host and in transovarial transmission is limited. In the majority of studies, for example, *Nosema heliothidis* infecting the hymenopteran *Campoletis sonorensis* (Brooks and Cranford, 1972), parasites have been observed in the adult female host reproductive tissue and then in host eggs, thus establishing that transovarial transmission occurs, but giving no detailed information on mechanisms. In this section we examine the mechanisms of transovarial transmission by microsporidia, the location of the parasites during host development and the mechanisms by which their life cycles are synchronized with host development and reproduction. We investigate the mechanisms by which microsporidia migrate to target gonadal tissue.

2.1. Localization of the Parasite during Host Development

Once transovarially transmitted to the host zygote, the parasite must find some strategy for survival in the host and transmission to target tissues. For those microsporidia which undergo continuous transmission from generation to generation, it is particularly important to minimize any detrimental effect on host reproduction and development whilst ensuring infection of the ova at the onset of host reproduction.

During embryogenesis the presence of large numbers of parasites throughout the host tissues may interfere with host development and survival and, therefore, would also be detrimental to the parasite. Various strategies have been developed to ensure safe passage of both parasite and host during this sensitive period of the host life cycle. First, some species of microsporidia are restricted to the yolk area of the embryo where they do not interfere with host development (Sajap and Lewis, 1988; Kellen and Lindegren, 1973). For example *Nosema* sp. is transovarially transmitted in the eastern tent caterpillar, *Malacosoma americanum*. When the larval host emerges the yolk is located in the midgut and parasite invasion occurs across this tissue (Nordin, 1975). Alternatively, parasites present in embryonic cells may be restricted to a specific area of those cells during host embryogenesis (Raina *et al.*, 1995; Terry *et al.*, 1999a). Parasite replication is often reduced or even stopped during this period thus reducing both the metabolic burden on the embryonic host and the chances of interference with embryogenesis (Kellen and Lindegren, 1973).

In some host/parasite systems the parasite location and morphogenesis change during host embryogenesis. *Nosema locustae* is transovarially transmitted to the embryos of the migratory locust, *Locusta migratoria migratorioides* and sporulation does not occur until embryogenesis begins. At the blastokinesis stage of embryonic development, spores were observed in the yolk whilst only meronts were found in the embryonic tissues (Raina *et al.*, 1995). *Nosema granulosis* is transovarially transmitted to the embryos of the amphipod, *Gammarus duebeni*. This parasite appears to use a very different strategy from other microsporidian parasites. Sporulation of *N. granulosis* does not occur during the embryogenesis of the host. Meronts of the parasite are located in the perinuclear cytoplasm of embryonic host cells, and are not restricted to the yolk. The parasites are present in relatively high numbers and are found in the majority of cells in the early stage embryo. However, by the time the host hatches the parasites are found in low numbers and are restricted to the host subcuticular cells. It is possible that the high parasite numbers in early embryogenesis allow the parasites to ensure that they are present in the target tissue. The large reduction in parasite numbers and the restriction of the infection to gonadal tissue in the adult host may reflect parasite death in non-target host cell lines (Terry *et al.*, 1997; Dunn *et al.*, 1998).

Two main paths of transmission to the reproductive tissue can be identified. The first path involves the targeting of the host reproductive tissue early in host development, with the parasite then remaining in this location throughout the life of the host (Dunn *et al.*, 1995; Terry *et al.*, 1997). In the second path the permanent site of infection is non-reproductive tissue and targeting of the ovarian tissue occurs immediately prior to host reproduction and oocyte maturation (Andreadis, 1983; Becnel *et al.*, 1987, 1989).

Early infection of the host ovarian tissue is seen in *N. granulosis* where infection occurs during host development. The parasites are located in the sub-epidermal cells of juvenile *G. duebeni*; they move to the follicle cells once the differentiation of the ovarian tissue has begun. *N. granulosis* then permanently resides in the follicle cells throughout the adult life of the host and invasion of developing oocytes occurs at each reproductive cycle from this location (Terry *et al.*, 1997). *Nosema plodiae* targets the gonad early in development of its host, the Indian meal moth, *Plodia interpunctella*. This parasite invades and multiplies in nurse cells and is passed into the oocytes through intercytoplasmic connections so that by adult emergence the percentage of transovarial transmission that will occur is already determined. Once in the oocyte, parasite development appears to be arrested until after fertilization and tissue differentiation has begun (Kellen and Lindegren, 1973). Early targeting of the ovarian tissue ensures transovarial transmission but may increase the chances of damaging the host reproductive tissue and, therefore, reduce transmission potential (Dunn *et al.*, 1995).

Late infection of the host reproductive tissue may prevent interference with the host reproductive cycle and reflect a damage limitation strategy by the parasite. However, transovarial transmission may be less efficient under this mechanism. Targeting of the gonad at or near to the time of host reproduction is observed in many insect hosts. *Amblyospora* sp., *Culicospora magna* and *Edhazardia aedis* infect mosquito hosts and establish a permanent site of infection in the host oenocytes. Sporulation and ovary invasion only occurs once the reproductive cycle of the host is initiated (Andreadis, 1983; Becnel *et al.*, 1987, 1989). Andreadis (1983) found that spores of *Amblyospora* sp. infecting *Aedes cantator* were present in oenocytes by the time the egg follicles had developed, although the mechanism of oocyte invasion is unclear. Spores of *Culicospora magna* are released into the body cavity of *Culex restuans* females following disintegration of the oenocytes and go on to infect oocytes from this site (Becnel *et al.*, 1987).

2.2. Timing of Parasite Development and Movement

In order to invade host tissues at a specific time point, it is necessary for the parasite to synchronize its life cycle with that of the host. It appears that the

parasite may react to hormonal signals produced by the host. Microsporidia of the genera *Nosema* (Canning and Hulls, 1970) and *Amblyospora* (Andreadis, 1983; Dickson and Barr, 1990) in mosquito hosts remain in the host oenocytes until a blood meal is taken. Following a blood meal, oocyte maturation takes place and it is at this point that the parasite invades the ovary. Parasite invasion appears to be stimulated by either the blood meal, or by the ensuing hormonal changes in the host. The autogenous mosquito, *Culex tarsalis* does not need a blood meal to stimulate ovarian development and studies of *Amblyospora californica* infecting this host demonstrate that a blood meal is not necessary for sporulation and ovary invasion, therefore suggesting that it is not the blood meal that the microsporidia are reacting to but a host reproductive event such as an underlying hormonal signal (Hall and Washino, 1986).

In *Gammarus duebeni* both the targeting of the ovarian tissue and the invasion of the oocytes by *Nosema granulosis* appear to occur at specific time points in the host's life cycle. In the juvenile host, parasites are found in the sub-epidermal cells and remain in this position until ovary differentiation begins, at which time they are observed in the newly differentiated follicle cells. Additionally, infection of the oocytes from the follicle cells only occurs once connections between these two cell types have formed and primary vitellogenesis has begun. This suggests that the parasites may be reacting to host hormones, for example those associated with gonadal differentiation and with oogenesis and vitellogenesis (Terry *et al.*, 1997).

2.3. Mechanisms of Parasite Migration within the Host

Parasite migration is the transfer of the parasite from one location within the host to another distinct location. Migration of microsporidia within the host can occur through three main mechanisms: spore germination (Iwano and Ishihara, 1991), infection of mobile host cells (Becnel *et al.*, 1989) and within cell distribution to daughter cells at host cell division (Terry *et al.*, 1999a).

Microsporidian spores contain a distinctive extrusion apparatus which enables them to penetrate and place the infective sporoplasm into host cells (Weidner, 1972; see Section 1). Spores are frequently involved with the infection of new hosts *per os* through germination and infection of the host midgut (Canning and Lom, 1986). However, a second spore type, the autoinfection spore, is also recognized in many microsporidia (e.g. Becnel *et al.*, 1987; Dickson and Barr, 1990). This autoinfection spore is involved in the cell to cell transfer of infection within a host individual (Iwano and Ishihara, 1991). In transovarially transmitted microsporidia the autoinfection spore may be used for gamete invasion (Terry *et al.*, 1997; see Section 1).

Direct invasion of the host oocytes has not been observed to date. However,

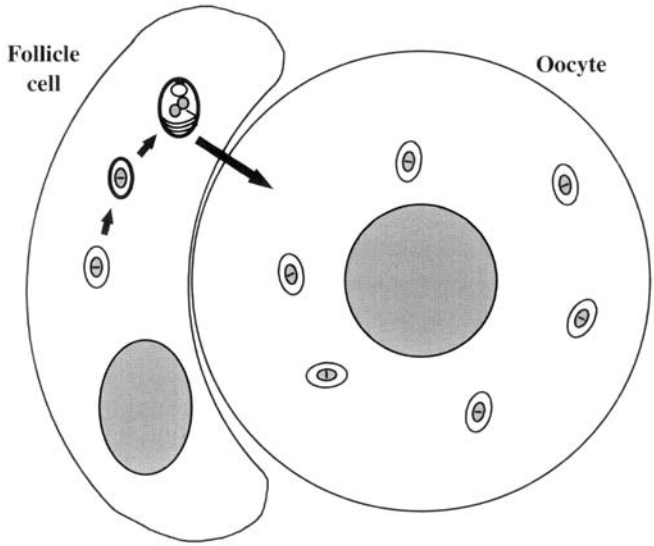


Figure 5 Invasion by *Nosema granulosis* of the host (*Gammarus duebeni*) gamete. Merogony and sporulation take place within the follicle cell which is the primary site of infection. Invasion of the host gamete takes place during vitellogenesis and may occur via cytoplasmic connections between the follicle cell and gamete or through direct penetration by the spore of the host cell membrane.

the arrangement of parasite stages within host ovarian tissue often suggests that this mechanism is utilized. In culicine mosquitoes, the transovarial transmission of microsporidia is often initiated when autoinfection spores germinate in or near to the ovaries and the sporoplasm is injected into the developing oocytes (Becnel, 1994). Similarly, the presence of empty *Nosema granulosis* spores within *Gammarus duebeni* follicle cells and of merogonic stages within oocytes suggest that spore germination results in direct invasion of the developing oocytes (Figure 5; Terry *et al.*, 1997). In some cases however, germination causes the infection of cells surrounding the ovarian tissue and the parasites then enter the oocytes by other means. Sporulation of *Amblyospora* sp. occurred prior to the infection of the ovaries of *Culex salinarius* and sporoplasms were then observed in the oenocytes and fat body. Andreadis and Hall (1979) suggested that sporoplasms entered the oocytes non-selectively during the transfer of vitellogens through pinocytosis. However, it seems difficult to envisage the transfer of such large parasites by this mechanism.

Movement within the host would be an extensive process in some host/parasite systems, if the parasite had to undergo sporulation and germination to cross each cell boundary between the permanently infected tissue and the oocytes. In the insect hosts in which parasites permanently reside in non-reproductive cells, movement to the ovarian tissue often occurs within mobile host cells. In mosquito hosts, microsporidia are found within oenocytes (secretory cells that undergo changes related to the moult cycle), which move to the area of the ovarian tissue at the time of oocyte development (Canning and Hulls, 1970; Becnel *et al.*, 1989). The microsporidia then sporulate and germinate to invade the ovaries from these cells. *Edhazardia aedis* and *Amblyospora* spp. multiply in the oenocytes of their mosquito hosts, the oenocytes come to lie near the host ovaries and the presence of empty spores suggests that oocyte invasion occurs from this location (Andreadis and Hall, 1979; Becnel *et al.*, 1989; Dickson and Barr, 1990). The use of mobile host cells is also seen in other insect hosts. *Nosema trichoplusiae* meronts, for example, multiply within the haemocytes of the cabbage looper, *Trichoplusia ni* and are transported to the ovarian tissue by the circulation of the haemolymph (Tanabe and Tamashiro, 1967).

Parasite movement within the host does not necessarily involve the parasite leaving one host cell and entering another. Microsporidia are present in the cytoplasm of the host cell. When a host cell divides the cytoplasm is split between the two daughter cells and the parasites may be present in one or both the daughter cells. The transovarially transmitted microsporidian *N. granulosis* is present throughout the early embryo of its *G. duebeni* host and there is evidence for differential segregation to target host cell lineages as development proceeds (Dunn *et al.*, 1998; Terry *et al.*, 1999a). The close association between the parasite and the host cell cytoskeleton in embryonic cells suggests that the parasite is using the host cytoskeleton to target daughter cells (see Section 1).

3. TRANSMISSION STRATEGIES AND PARASITE MAINTENANCE

Microsporidian life cycles are complex, can involve a number of transmission routes and may require more than one host species. For the majority of microsporidian parasites, horizontal transmission is the major transmission route (Canning and Lom, 1986). However, for many microsporidia of invertebrates, transovarial transmission is also important for parasite maintenance in the host population. In this section, we review the transmission strategies employed by these microsporidia and their relationship to the host life cycle and ecology.

3.1. Horizontal Transmission

Horizontal transmission represents the major transmission path utilized by the majority of microsporidia infecting invertebrate hosts (Figure 6). Where horizontal transmission is the sole or primary transmission route, infection is often associated with high mortality as a result of parasite replication and metabolic load (Becnel *et al.*, 1995). In general microsporidia undergo extensive proliferation within the host tissue, resulting in the production of huge numbers of spores and causing massive tissue destruction (Lange *et al.*, 1996). Microsporidia are released from the host through faeces (de Graaf *et al.*, 1994) and secretory products (Canning and Hulls, 1970), or are released on the death of the host (Lange *et al.*, 1996). These spores are then transmitted *per os*, through ingestion by a new host individual and invasion of the host tissues occurs across the midgut (Becnel, 1994; Smallridge *et al.*, 1995). Many horizontally transmitted parasites are present in carnivorous insects and chewing insects that ingest horizontal spores through their natural feeding patterns (e.g. Lange *et al.*, 1996).

3.2. Transovarial Transmission

In a small number of microsporidia, transovarial transmission appears to be the sole route of transmission to new hosts (Figure 7; Bulnheim and Vavra, 1968; Bulnheim, 1971; Dunn *et al.*, 1993; Ni *et al.*, 1997; Terry *et al.*, 1997) and transmission is intricately associated with the host life cycle and ecology (see Section 4). The two most well studied examples of these microsporidia are both found within the genus *Nosema* (Ni *et al.*, 1997; Terry *et al.*, 1998; 1999b). *Nosema empoascae* is transovarially transmitted in its host the potato leaf hopper (*Empoasca fabae)* and horizontal transmission through host death,

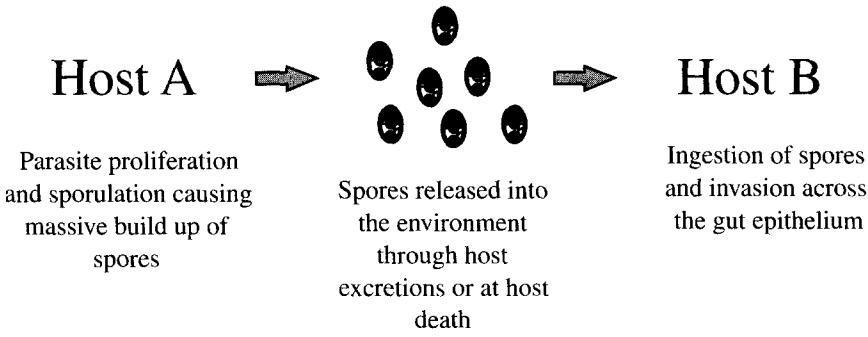


Figure 6 Diagrammatic representation of horizontal transmission.

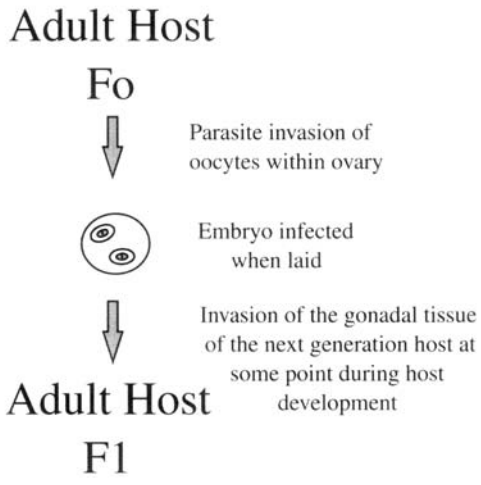


Figure 7 Diagrammatic representation of transovarial transmission.

excretion and contact have not been observed. The restriction of this parasite to the transovarial transmission route may reflect the feeding mechanism of its host. *E. fabae* has piercing and sucking mouthparts and feeds on plant saps and is therefore unlikely to encounter environmental spores in the same way as an insect with chewing mouthparts (Ni *et al.*, 1997).

Nosema granulosis infecting *Gammarus duebeni* also appears to be solely transovarial transmitted, with no evidence for horizontal or paternal transmission (Terry *et al.*, 1998). In contrast to microsporidia in those mosquito hosts found in still water habitats, *G. duebeni* inhabits fast flowing water and is restricted to the intertidal region. Horizontal parasite transmission would be very inefficient in this habitat as spores would be swept away from potential new hosts.

3.3. Mixed Transmission Strategies

Many microsporidia exhibit both horizontal and transovarial transmission and the strategies employed by these parasites depend on host ecology and the opportunities for transmission and dispersal. Mixed transmission (Figure 8) is seen for a variety of species infecting mosquitoes including *Amblyospora* spp. (Andreadis and Hall, 1979; Lord *et al.*, 1981), *Edhazardia aedis* (Becnel *et al.*, 1989) and *Parathelohania anophelis* (Avery and Undeen, 1990). Transovarial

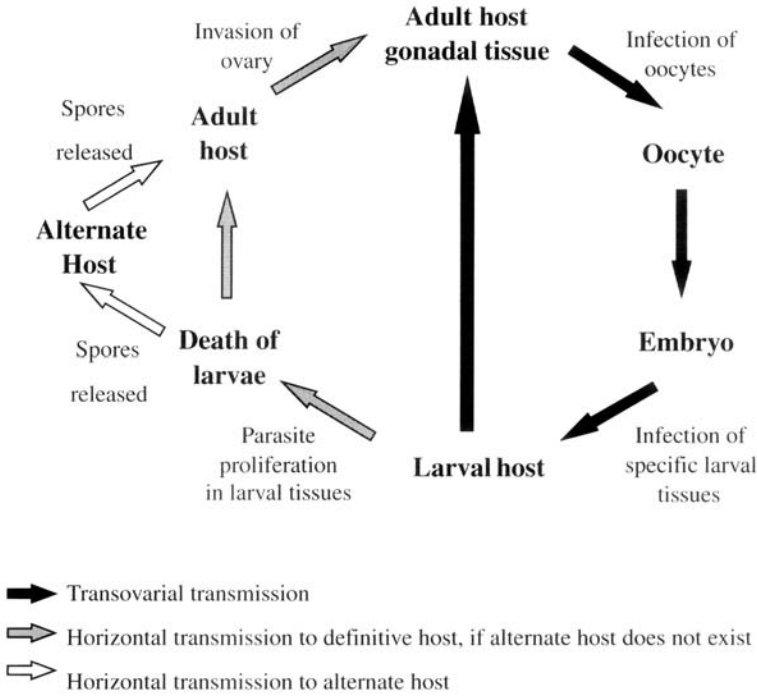


Figure 8 Diagrammatic representation of mixed transmission.

transmission may occur through successive host generations (Andreadis, 1983; Becnel *et al.*, 1989) or there may be obligate alternation between the transovarial and horizontal transmission cycles (Figure 8; Lord *et al.*, 1981). Whilst some parasites have a direct life cycle, others require an intermediate, copepod host (e.g. Sweeney *et al.*, 1990).

E. aedis is both horizontally and transovarially transmitted and requires two generations of the mosquito host *Aedes aegypti* to complete its life cycle (Undeen *et al.*, 1993). The parasite is transovarially transmitted from mother to offspring and a build up of spores in the larval host results in larval death and subsequent horizontal transmission to a new mosquito host individual (Becnel *et al.*, 1989). Other members of the family Amblyosporidae have an indirect life cycle. *Amblyospora indicola* undergoes a similar transovarial cycle to *E. aedis* and build up of spores in the larval host. However, spores released from the larva are not infective to other mosquitoes but are infective to an alternate copepod host, *Apocyclops* sp. (Sweeney *et al.*, 1990).

Transovarial transmission may permit parasite maintenance when host densities are low and environmental conditions are unfavourable (Lucarotti and

Andreadis, 1995). For example, *Amblyospora* spp. infecting northern populations of *Aedes* overwinter in the diapausing host eggs (Andreadis, 1985b, 1990). Similarly, Canning *et al.* (1985) found that microsporidia infecting the winter moth *Operophtera brumata* survived in the host eggs during long (9 month) periods of host inactivity. For other microsporidia, survival may be achieved through the use of an alternate host. For example, populations of *Culex dolosus* drop at extreme temperatures in summer and the microsporidian *Amblyospora dolosi* is maintained in its alternate copepod host until the mosquito population rises again in the autumn (Micieli *et al.*, 1998).

Transovarial transmission can also provide a mechanism for long range dispersal of microsporidia to new habitats (Lucarotti and Andreadis, 1995). *E. aedis* and *Amblyospora* spp. infect mosquito larvae which are found in patchy, often ephemeral aquatic habitats. Infected adult hosts may disseminate these parasites and death of their transovarially infected young will release the parasite into new aquatic environments (Becnel *et al.*, 1995; Lucarotti and Andreadis, 1995).

Microsporidia display genetic variation for transmission strategy and life cycle. Sweeney *et al.* (1989) carried out selective breeding from mosquito hosts harbouring transovarial type spores of *Amblyospora dyxenoides*. Selection resulted in an increase in the proportion of transovarial infections in the population and a decrease in the horizontal, fat body infections. Similarly, Iwano and Kurtti (1995) selected for transovarial type spores of *Nosema furnacalis* maintained in cell culture. They found that the frequency of transovarial spores (associated with autoinfection) increased from 50% to 100% following repeated passages in cell culture, suggesting that restriction of transmission to one route may eventually lead to the loss of the alternate spore type.

Koella *et al.* (1998) demonstrated phenotypic plasticity of the development and transmission route of *E. aedis*. Under good conditions, when the host is likely to survive and reproduce, *E. aedis* has alternating horizontal and transovarial transmission. However, high parasite burden and low food availability decreases host reproduction and hence opportunities for transovarial transmission. Under such poor host conditions, Koella *et al.* (1998) observed an increase in condensed parasite development and repeated horizontal transmission. The parasite was able to condense its life cycle in response to limited opportunities for transovarial transmission.

4. THE ECOLOGY AND EVOLUTION OF TRANSOVARIALLY TRANSMITTED MICROSPORIDIA

Transovarially transmitted microsporidia can shed light on some key areas in the evolutionary ecology of parasitism. The use of both horizontal and transovarial transmission routes provides an opportunity to examine current theories

for the evolution of virulence. In addition, transovarially transmitted microsporidia can manipulate their hosts through sex ratio distortion. We consider the implications of parasitic sex ratio distortion for host population sex ratio and stability.

4.1. The Evolution of Virulence in Transovarially Transmitted Microsporidia

Horizontally transmitted microsporidia, such as *Nosema apis*, typically cause massive infections in the host, are pathogenic and may lead to host mortality (Canning, 1993). Horizontal transmission is strongly dependent on parasite burden as spores are released into the environment where they are ingested by the next host. The more spores produced, the greater the chance of infecting a new host. Associated with massive spore production is a high metabolic cost and tissue damage in the host leading to a reduction in host fitness and survival (Table 2).

Table 2 The relationship between transmission strategy and virulence amongst some microsporidia of insect hosts.

Parasite	Primary transmission route	Virulence	Reference
Nosema apis	Horizontal	High: reduced fecundity and activity, increased mortality.	Canning, 1993
Edhazardia aedis	Alternating horizontal and vertical	*Vertical phase* Low: slight reduction in fecundity *Horizontal phase* High: larval mortality	Koella and Agnew, 1997 Becnel, 1995
Nosema granulosis	Vertical	Low: fecundity slightly reduced, survival unaffected.	Terry *et al.*, 1998

In contrast, transovarially transmitted microsporidia are frequently present in low burden and cause little pathogenicity (Table 2, Dunn *et al.*, 1993; Terry *et al.*, 1997; Koella and Agnew, 1997; Ni *et al.*, 1997). Vertically transmitted parasites rely on successful host reproduction for their transmission to the next host generation. Any negative effects on host fitness (and hence reduction in reproductive success) will result in a reduction of potential new hosts for infection. Hence selection is expected to favour reduced virulence in vertical host–parasite associations (Ewald, 1987; Smith and Dunn, 1991; Dunn *et al.*, 1995) and this

prediction has been supported by empirical studies of bacteria/phage (Bull *et al.*, 1991) and fig wasp/nematode associations (Herre, 1993).

There are conflicting selective pressures on parasite burden and associated virulence. Transovarial transmission is dependent on successful infection of the host gametes which, in turn, depends upon parasite burden (Hurst *et al.*, 1994; Dunn *et al.*, 1995). However, any reduction in host fitness resulting from the parasite burden will be selected against. The low level of pathogenicity caused by transovarially transmitted microsporidia represents a trade-off between the conflicting selective pressures for high transmission and low virulence, and some microsporidia have evolved very precise and complex mechanisms of gamete infection (Dunn *et al.*, 1998; Terry *et al.*, 1998, Section 2) in which relatively low parasite numbers are required to ensure infection of host gametes.

Microsporidia for which transovarial transmission is the sole or major route have been shown to cause little or no reduction in host fitness. For example, Bulnheim and Vavra (1968) found that *Octosporea effeminans* was present in very low burden in its *Gammarus duebeni* host and caused no discernible pathogenicity. Female growth, moult frequency, fecundity and survival were all unaffected by the parasite. Terry *et al.* (1997) found that *Nosema granulosis* in the same crustacean host caused limited pathogenicity. Host survival was unaffected by the parasite. However, the growth rate of infected young was reduced leading to low adult weight and a reduction in fecundity of about 25%. Similarly, Ni *et al.* (1997) found little disease induced mortality in the leaf hopper *Empoasca fabae* infected with *Nosema empoascae* and reported an increase in the egg production of infected, transmitting females.

In contrast, higher levels of virulence during the transovarial phase of the life cycle have been reported for those microsporidia which have alternating horizontal and transovarial transmission routes. Fecundity, egg hatch and adult emergence were greatly reduced in female *Aedes aegypti* mosquitoes which were infected with *Edhazardia aedes* as larvae and then went on to transmit the infection to their progeny (Becnel *et al.*, 1995). Andreadis and Hall (1979) reported a 50% reduction in egg hatch of *Culex salinarius* infected with transovarially transmitting *Amblyospora* spp. The higher pathogenicity in parasites with mixed versus sole transovarial transmission may reflect weaker selection for low pathogenicity where horizontal transmission provides an alternate route to new hosts.

Alternatively, the higher virulence may reflect competition between different parasite strains (Hurst *et al.*, 1994). Models for the evolution of virulence assume strict clonality of vertically transmitted parasites (Ewald, 1987) and microsporidia which are solely transovarially transmitted would be expected to be strongly clonal, as novel strains within a host lineage can only occur through mutation. However, for those microsporidia which have alternating horizontal and transovarial routes, hosts may become infected *per os* with more than one parasite strain. The different strains will therefore be in competition during the

transovarial phase. The observed differences in the virulence of clonal versus less-clonal microsporidia during the transovarial phase of the life cycle may provide the first evidence supporting the prediction of Hurst *et al.* (1994) of higher virulence under inter-strain competition. Direct measurements of virulence in relation to clonality are required to test this hypothesis more fully.

Microsporidia with alternating transmission cycles show markedly different levels of virulence during transovarial and horizontal transmission cycles (Table 2). During the transovarial phase, *Amblyospora* sp. replicates but causes little pathology in the mosquito host. Fecundity, longevity and the onset of oviposition were unaffected by the parasite (Andreadis and Hall, 1979; Andreadis, 1983, 1985b; Sweeney *et al.*, 1989) In contrast, during the horizontal phase, the parasite causes a massive infection of the fat body killing the larva and releasing spores for transmission to the intermediate copepod host (Lucarotti and Andreadis, 1995). Similarly, *E. aedis* causes little pathogenicity in the transovarial phase but is lethal in the horizontal phase (Becnel *et al.*, 1995; Koella and Agnew, 1997).

It is interesting that in all the *Thelohania, Amblyospora* and *Edhazardia* species studied pathology is severe, but delayed and host death occurs during the fourth larval instar (Kellen *et al.*, 1965, 1966; Becnel, 1994; Lucarotti and Andreadis, 1995). Andreadis (1983) observed little or no multiplication of *Amblyospora* within embryos of transovarially infected eggs. In addition, development of horizontal spores is localized in the fat body of hosts infected with *E. aedis* (Becnel, 1994) and *Amblyospora* spp. (Lucarotti and Andreadis, 1995). These may all be strategies to maximize horizontal transmission. Delaying host death may maximize the time and resources available for parasite replication and the fourth instar is the last host stage from which microsporidia can escape into the water and target their next host (Hurst, 1991). However, Lucarotti and Andreadis (1995) point out that localization of the infection in fat tissue may also limit the potential for horizontal transmission and, in contrast with those microsporidia which develop in the gut, horizontal transmission of *Amblyospora* can only occur following host death.

Studies of transmission and virulence amongst the microsporidia provide rare empirical evidence to support theoretical predictions for virulence evolution (Ewald, 1987; Bull *et al.*, 1991; Smith and Dunn, 1991) and provide valuable model systems for the further development of this area of evolutionary biology.

4.2. Parasitic Sex Ratio Distortion, a Strategy of Transovarial Parasites

Transovarial transmission is generally a highly efficient transmission route for microsporidia and other cytoplasmic parasites (e.g. Dunn *et al.*, 1995).

However, it has been shown theoretically that transovarial transmission alone cannot sustain a parasite in the host population in the absence of some mechanism to increase the relative frequency of infected females (Fine, 1975; Anderson and May, 1981; Werren, 1987; Hatcher and Dunn, 1995). A number of elegant strategies have evolved in transovarially transmitted parasites (reviewed in Dunn *et al.*, 1995; O'Neill *et al.*, 1997) which increase the relative frequency of the transmitting, female sex (Table 3). Two of the strategies summarized below, late male killing and feminization, have been reported for microsporidia infecting arthropod hosts.

Table 3 Strategies used by transovarially transmitted parasites to enhance their spread through the host population, illustrated by example systems.

Strategy	Host	Parasite	Reference
Metabolically advantageous to the host	Aphids	*Buchnera*	Douglas and Prosser, 1992
Cytoplasmic incompatibility	*Drosophila* spp.	*Wolbachia*	Hoffman and Turelli, 1998
Induction of parthenogenesis	*Trichogramma* spp.	*Wolbachia*	Stouthamer, 1998
Primary sex ratio distortion	*Nasonia vitripennis*	Unidentified 'maternal sex ratio factor'	Werren, 1987
Early male killing	*Adalia bipunctata*	*Rickettsia*	Hurst *et al.*, 1992
Late male killing	Mosquito species	Microsporidia	Kellen *et al.*, 1965
Feminization	*Armadillidium vulgare*	*Wolbachia*	Rigaud, 1997
	Orchestia gammarellus	*Paramartelia*	Ginsburger-Vogel, 1980
	Gammarus duebeni	Microsporidia	Bulneim and Vavra, 1968

Parasites such as bacterial mycetocyte symbionts of aphids can enhance transmission through the host population by imparting a metabolic advantage on their host (e.g. Douglas and Prosser, 1992). In mosquitoes and *Drosophila*, cytoplasmic incompatibility increases the frequency of rickettsia-like bacteria by causing death of uninfected females and a consequent increase in the frequency of infected hosts (e.g. Yen and Barr, 1973; O'Neill and Karr, 1990). Parthenogenesis is induced in haplodiploid wasps by maternally inherited *Wolbachia*. The parasite prevents chromosomal separation during mitosis of the zygote thus converting haploid (male) zygotes to diploids and increasing the relative frequency of transmitting female hosts (Stouthamer *et al.*, 1990,

1993; Stouthamer and Kazmer, 1994). Primary sex ratio distortion is caused by an unidentified sex ratio distorter in the haplodiploid wasp *Nasonia vitripennis* which increases the number of fertilized (female) eggs (Skinner, 1982; Werren, 1987).

The term male-killers refers to transovarially transmitted microparasites which cause sex-specific mortality in their hosts. Infection is benign in infected females, the hosts develop normally and go on to transmit the parasite to subsequent generations. However, male-killers are pathogenic to their male hosts, infection leading to mortality during host development (reviewed in Skinner, 1985; Hurst, 1991; Hurst *et al.*, 1997). There are two groups of male-killing microparasites: early male-killers and late male-killers. Early male-killing is a strategy employed by a range of bacterial parasites, which enhances parasite prevalence indirectly through release of resources to female sibs containing clonal relatives of the parasite (Skinner, 1985; Hurst, 1991) or through decreasing sib–sib antagonism or inbreeding (Werren, 1987; Hurst *et al.*, 1997). Late male-killing, on the other hand, increases parasite prevalence directly through horizontal transmission from the dead host and this strategy is used by a number of microsporidia infecting mosquitoes (Kellen *et al.*, 1965; Skinner, 1985; Hurst, 1991). Finally, feminization of the host directly increases the numbers of the transmitting sex through conversion of genotypic males to phenotypic females. Feminization has been reported for bacteria, haplosporidia and microsporidia infecting a number of arthropod hosts (Bulnheim and Vavra, 1968; Martin *et al.*, 1973; Ginsburger-Vogel and Desportes, 1979; Kageyama *et al.*, 1998).

4.2.1. *Male-killing Microsporidia*

Late male-killing has been reported for several species of *Amblyospora* and *Thelohania* infecting mosquito hosts (Kellen *et al.*, 1965; Hurst, 1991). These parasites display sex-differential development and virulence in their hosts (Figure 9). In female hosts, the infection is benign and is restricted to the oenocytes. Vertical type spores are produced which are transovarially transmitted to the next generation of hosts and females of this F1 generation also have benign infections. However, in F1 generation male hosts (i.e. infected vertically) a different sporulation sequence occurs. Massive parasite replication in the fat body results in death during the fourth larval instar and subsequent release of spores into the environment for horizontal transmission.

Varying degrees of male-killing have been reported amongst microsporidia which Kellen *et al.* (1965) designated types 1–4. True male-killers (type 1 infections, Kellen *et al.*, 1965) are typified by *Amblyospora californica* (=*Thelohania californica*) which kills transovarially infected males but forms

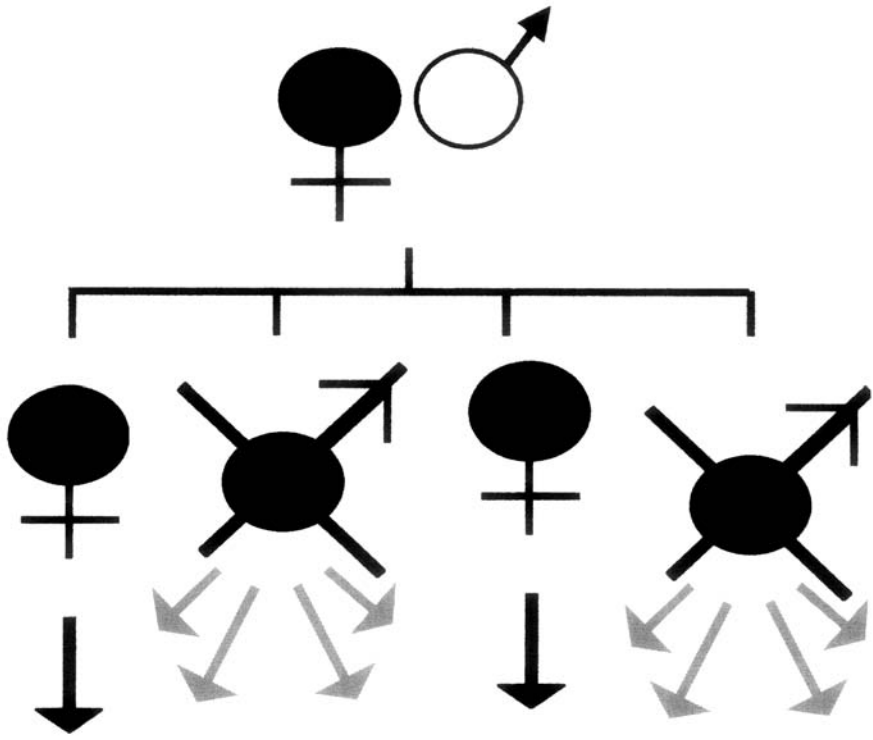


Figure 9 Late male-killing. Male-killing microsporidia form a benign infection in the female host which survives to transmit the parasite transovarially (black arrows). A massive infection is produced in the male host which leads to host death at the fourth instar and subsequent release of horizontal type spores into the environment (grey arrows).

a benign infection in females which then mature and transmit the parasite to the next generation of hosts. In contrast, most hosts which inherit *Amblyospora campbelli* (type 3 infection) die and there is little sex-specific mortality, although the few surviving females transmit the parasite to their progeny.

Hurst (1991) and Sweeney *et al.* (1990) suggested that the different degrees of sex-specific mortality amongst microsporidia reflect the relative efficiency and importance of horizontal versus transovarial transmission in the life cycle of the different species. There is no selective advantage to parasites causing a benign infection in males as there is no paternal vertical transmission, and male mortality will be selected if spores are released for horizontal transmission. In female hosts, virulence should be selected if the opportunities for horizontal

transmission are high, whereas a benign infection should be selected if transovarial transmission is important for parasite maintenance. For example, larval death of male and female mosquitoes infected with *A. campbelli* releases spores which infect a copepod intermediate host (Dickson and Barr, 1990). In contrast, transovarial transmission is necessary to maintain *A. californica* outside of the mosquito breeding season and this parasite produces benign, transovarial spore types in the female host (Kellen *et al.*, 1965).

4.2.2. *Feminizing Microsporidia*

Feminizing microparasites distort host sex ratio by converting genotypic males into functional, phenotypic females (Juchault *et al.*, 1992; Dunn *et al.*, 1995). These parasites are solely transovarially transmitted. Feminization converts non-transmitting male hosts into females capable of transmitting the parasite to future generations of hosts, thus increasing the relative frequency of infected

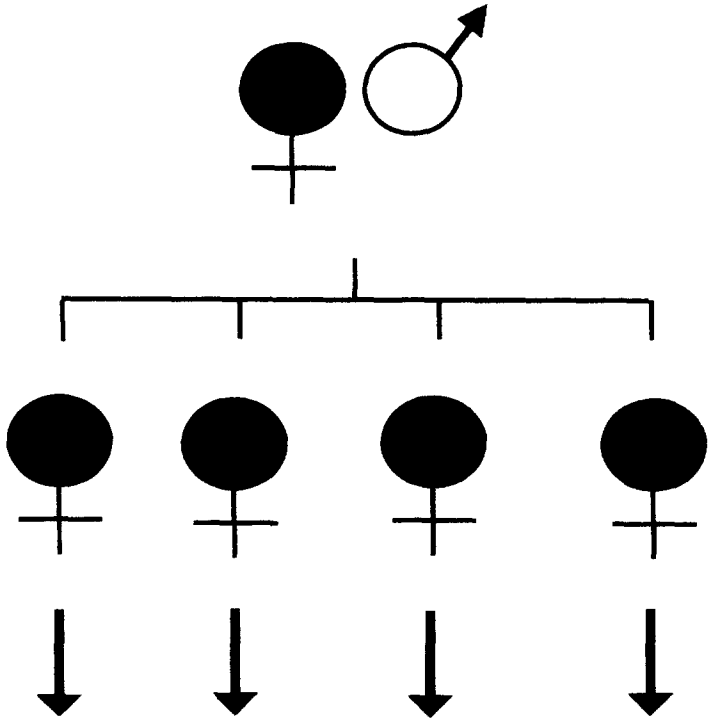


Figure 10 Feminization. Feminizing parasites convert genotypic male hosts into phenotypic females which transmit the parasite transovarially to the next generation of hosts.

females in the population (Figure 10, Hurst, 1993; Dunn *et al.*, 1995). The majority of feminizers have been reported from crustacean hosts, although a feminizing bacterium has recently been reported in the Asian corn-borer *Ostrinia furnacalis* (Kageyama *et al.*, 1998). Amongst crustaceans, feminization is caused by *Wolbachia* infecting several species of isopods (Martin *et al.*, 1973; Rousset *et al.*, 1992; Rigaud *et al.*, 1997) by *Paramartelia orchestiae*, a haplosporidian-like parasite infecting the amphipod *Orchestia gammarellus* (Ginsburger-Vogel and Desportes, 1979) and by a number of microsporidia infecting the amphipod *Gammarus duebeni*.

At least four, ultrastructurally distinct species of feminizing microsporidia have been reported infecting the crustacean *G. duebeni*. Bulnheim and Vavra (1968) first described a feminizing microsporidian, *Octosporea effeminans* from the Elbe estuary, Germany. Bulnheim (1971) also described *Thelohania hereditaria* from the Elbe estuary and more recent studies have identified a further two feminizing microsporidia infecting this host (Table 4). These parasites caused little or no pathogenicity in their hosts (Section 3). However, breeding experiments showed that they had a strong feminizing effect on the host (Bulnheim and Vavra, 1968; Bulnheim, 1971; Dunn *et al.*, 1993; Terry *et al.*, 1998). As a result, the majority of the young of infected mothers were female (Figure 11). Artificial infection experiments with *O. effeminans* and *Nosema granulosis* confirmed that these microsporidia were the agents of feminization in the host (Bulnheim, 1977; Dunn and Rigaud, 1998). Microsporidian induced feminization is also suspected in other crustacean hosts (A.M. Dunn *et al.*, unpublished data).

The restriction of feminizing microsporidia to crustacean hosts may reflect the sparsity of studies of transovarially transmitted parasites. Lack of pathogenicity means that such infections will only be found through epidemiological study or through observations of sex ratio distortion in the host. Nonetheless, it is surprising that parasitic feminization is so widespread in crustacea and that *G. duebeni* is host to several feminizers. Sex differentiation in crustacea occurs after the second moult and is controlled by the androgen gland which produces

Table 4 Feminizing microsporidia infecting *Gammarus duebeni*. Prevalence data refers to the percentage of infected females.

Parasite	Host population	Prevalence	Reference
Octosporea effeminans	Elbe Estuary, Germany	30%	Bulnheim and Vavra, 1968
Thelohania hereditaria	Elbe Estuary, Germany	7%	Bulnheim, 1971
Unidentified	Northumberland, UK	31%	Dunn *et al.*, 1993
Nosema granulosis	Isle of Cumbrae, UK	46%	Terry *et al.*, 1999

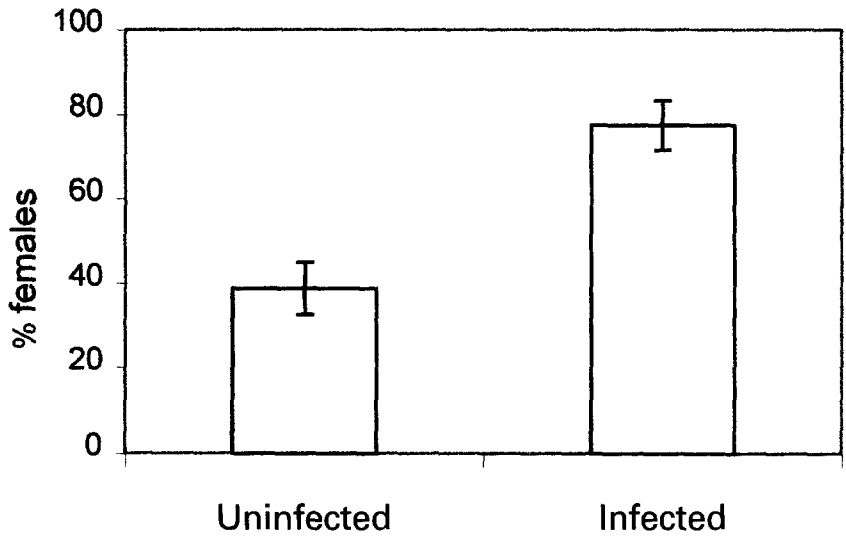


Figure 11 The effect of the feminizing microsporidian *Nosema granulosis* on the sex ratio of *Gammarus duebeni* broods. The graph shows sex ratios produced by 82 uninfected females and 63 infected females. There are significantly more females in infected broods than in uninfected broods.

androgen hormone (Katakura, 1984; Charniaux-Cotton and Payen, 1985). If the androgen gland differentiates, male development occurs; in the absence of androgen gland differentiation, female development takes place. The lability of sex determination in the Crustacea may permit feminization and Bulnheim (1977) and Rigaud (1997) suggest that these feminizers suppress androgen gland development and androgen hormone reception to induce feminization of the host.

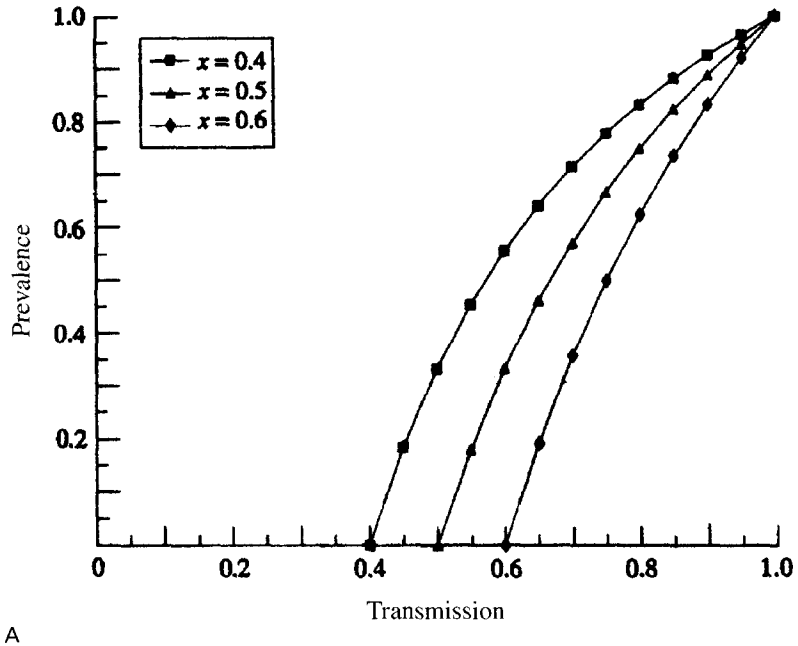
It has also been suggested that environmental sex determination (ESD) may increase vulnerability of *G. duebeni* to microsporidian feminization (Dunn *et al.*, 1995). ESD is an adaptive sexual strategy under which the sex of an individual is matched to its future size related fitness according to environmental cues (Adams *et al.*, 1987). The level of ESD varies between *G. duebeni* populations (Watt and Adams, 1993) and it is interesting to note that a study of prevalence of the third, unidentified microsporidian feminizer (Table 4) across a series of populations found that parasite prevalence was higher in those populations with a high level of ESD. This supports the idea that the delay in sex determination under ESD makes *G. duebeni* vulnerable to parasitic manipulations of sex. The resulting increase in the frequency of the transmitting (female) sex may permit invasion of sex ratio distorters into the host population (Dunn *et al.*, 1995).

Microsporidian feminization may have implications for the ecology and evolution of *G. duebeni*. As a feminizing microsporidian spreads through the host population, the sex ratio will become more female biased selecting for host resistance to parasite transmission and feminization (Taylor, 1990) and for compensatory sex ratio evolution (Werren, 1987; Hatcher and Dunn, 1995) as well as affecting host population stability and extinction (Hatcher *et al.*, 1999a,b).

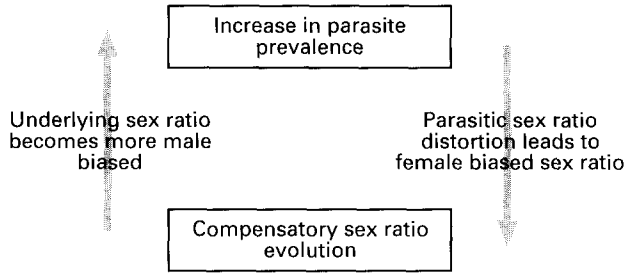
The transmission efficiency of *Nosema granulosis* has been shown to vary between broods from different host mothers (Terry *et al.*, 1998). Similarly, interfamily differences in parasite transmission efficiency have been reported for other microsporidian feminizers infecting *G. duebeni* (Bulnheim, 1978; Dunn *et al.*, 1993; Terry *et al.*, 1998) and interfamily differences in feminization efficiency have been reported for *O. effeminans* and *T. hereditaria*. These differences may reflect clonal differences between parasite strains in the different host lineages or they may result from differences in host susceptibility or resistance to parasite transmission, growth and feminization (Dunn and Hatcher, 1997).

The sex ratio bias induced by feminization may lead to conflict between parasite and host over sex ratio. Selection on the parasite will favour a female biased sex ratio since only females transmit the infection. However, selection acting on the host genes will generally favour a 1 : 1 primary sex ratio (Fisher, 1930). As a result, host autosomal genes which code for a greater investment in males (the rare sex) may be favoured (Werren, 1987; Hatcher and Dunn, 1995; Hatcher and Tofts, 1995) leading to a more male biased uninfected host sex ratio. However, prevalence is co-dependent on host sex ratio. Equilibrium parasite prevalence is higher when the uninfected host sex ratio is more male biased (Hatcher and Dunn, 1995). Co-evolutionary feedback between host sex ratio and prevalence can drive parasite prevalence upwards and can lead to the emergence of novel sex determining mechanisms in the host (Bull, 1983; Werren, 1987; Taylor, 1990; Hatcher and Dunn, 1995). This feedback between parasite prevalence and host sex ratio is predicted to lead to fixation of the parasite in females (all females are infected) and monogeny in the host (Figure 12A,B, all hosts being genetically male; Werren, 1987; Hatcher and Dunn, 1995).

In some *A. vulgare* populations, sex ratio evolution in the presence of a feminizing *Wolbachia* has gone to the limit as predicted theoretically. All individuals are genetically male and infected individuals are feminized by the bacteria which has become the sex determining factor in this host (Juchault *et al.*, 1993; Rigaud *et al.*, 1997). However, there is no evidence for parasite fixation by microsporidian feminizers. Feminization is not 100% efficient for these parasites (Terry *et al.*, 1997) and so the drive to monogeny and fixation will be less strong (Hatcher and Dunn, 1995). However, male biased sex ratios have been recorded in uninfected hosts from several *G. duebeni* populations



A



B

Figure 12 Co-evolutionary feedback between parasite prevalence and underlying host sex ratio. A. The effect of the sex ratio produced by uninfected hosts on parasite prevalence at equilibrium. Predicted equilibrium prevalence is plotted against parasite transmission rate for three different underlying (uninfected) host sex ratios (x, expressed as proportion of females). A more male biased underlying sex ratio results in a higher equilibrium prevalence. Parasite prevalence depends upon the host sex ratio which, in turn, depends upon parasite prevalence (Hatcher and Dunn, 1995). B. Co-evolutionary feedback between parasite prevalence and host ESS (evolutionary stable stategy) sex ratio can lead to increased parasite prevalence and a male biased (uninfected) host sex ratio and may even drive the host population to monogeny where all hosts are genetically male and female hosts result from parasite-induced feminization.

where *N. granulosis* prevalence is high (Terry *et al.*, 1997; Dunn, unpublished), which may reflect compensatory host sex ratio evolution in response to microsporidian sex ratio distortion.

Even if a feminizer does not go to fixation in the host population, theoretical models predict that the host population may be driven to extinction if males become too rare to sustain the population through reproduction (Hatcher *et al.*, 1999a). However, microsporidian sex ratio distorters appear to be relatively stable in natural *G. duebeni* populations (Dunn *et al.*, 1995) and recent models show that metapopulation structure may enable host/parasite coexistence through turnover of uninfected, infected and extinct patches (Hatcher *et al.*, 1999b).

5. CONCLUSIONS

Transovarial transmission is an important part of the life cycle of many microsporidia and recent information on the structure of the transovarial spore and its overlap with the autoinfection spore raise exciting questions about the origin of transovarial transmission. Future work on transovarial spore morphology and life cycles, in conjunction with a molecular taxonomic approach will increase our understanding of microsporidian phylogeny and evolution.

Transovarial transmission is typically associated with low virulence and so is often overlooked. Most examples have been noted following study of horizontal life cycle stages and there are few studies of microsporidia which rely solely on transovarial transmission. However, recent work suggests that this strategy may, in fact, be widespread in this phylum with the cellular relationship between parasite and host contributing to our understanding of the origin and evolution of endosymbiosis.

Despite the lack of classic pathogenic effects on the host, transovarially transmitted microsporidia may have profound effects on host population sex ratio and stability. Theoretical studies of virulence and sex ratio are expanding areas in evolutionary biology and these microsporidia provide a rare opportunity for empirical study and test.

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Adhesive Secretions in the Platyhelminthes

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ABSTRACT

This review is the first to draw together knowledge about bioadhesives secreted by a group of parasites. Mechanisms of mechanical attachment are well known among parasites, but some can also attach to host surfaces by chemical means using a thin layer of adhesive material secreted at the parasite–host interface. Attachment by adhesives to living surfaces has not been studied in detail previously. A significant volume of research has determined much about the chemistry and nature of bioadhesives secreted by various marine macroinvertebrates from different phyla for attachment to inert substrates. Mussels and barnacles are sessile and adhere permanently, whereas starfish display temporary but firm adhesion during locomotion, feeding and burrowing. We focus on the Platyhelminthes that comprises the largely free-living Turbellaria and the wholly parasitic Monogenea, Cestoda, Digenea and Aspidogastrea. The term *tissue adhesion* is introduced to describe attachment by adhesives to epithelial surfaces such as fish epidermis and the lining of the vertebrate gut. These living layers regenerate rapidly, secrete mucus, are a site for immune activity and are therefore especially hostile environments for organisms that inhabit them, presenting a significant challenge for adhesion. Not all platyhelminths adhere to living surfaces and types of adhesion to inert substrates by the free-living turbellarians are also reviewed. Tissue adhesion is particularly well exemplified by monopisthocotylean monogeneans, parasites that are especially mobile as larvae, juveniles and adults on the epidermis of the body and gill surfaces of fish. These monogeneans secrete adhesives from the anterior end when they move

from site to site, but some have secondarily developed adhesives at the posterior end to supplement or replace mechanical attachment by hooks and/or by suction. The temporary but tenacious anterior adhesives of monogeneans display remarkable properties of instant attachment to and detachment from their host fish surfaces. In contrast to the mobility of turbellarians and monopisthocotylean monogeneans and the simplicity of their direct life cycles, the largely endoparasitic Cestoda and Digenea are considered to be less mobile as adults. The complex cestode and digenean life cycles, involving intermediate hosts, place different demands on their various stages. Diverse, mostly anterior, gland cells in larvae, metacestodes and adults of the true tapeworms (Eucestoda), and in larval and adult Gyrocotylidea and Amphilinidea are reviewed. Conspicuous gland cells, mostly but not exclusively at the anterior end, in miracidia, cercariae and adults of digeneans and in cotylocidia and adults of aspidogastreans are also reviewed. Unlike turbellarians and monogeneans, accounts of unequivocal adhesive secretions in the Cestoda, but especially in the Digenea and Aspidogastrea, are relatively rare. The primary purpose of many conspicuous glands in the different stages of these mostly endoparasitic flatworms is for penetration into, or escape from, different hosts in their life cycle. We provide a detailed review of current knowledge about adhesion (in the sense of a thin layer of chemical material) in the Platyhelminthes including uses among eggs, larval, juvenile and adult stages. Information on structure, morphology and ultrastructure of the various adhesive systems that have been described is reviewed. Application of the 'duo gland' model is discussed. Comparisons are made between the little that is known about the chemistry of flatworm adhesives and the significant knowledge of the chemical nature of other invertebrate bioadhesives, especially those from marine macroinvertebrates. The potential importance of adhesives in parasitism is discussed. Phylogenetic considerations and evolutionary implications are covered and we conclude that insufficient structural and chemical evidence is available to determine homology or otherwise between the adhesive systems in turbellarians and monogeneans and between anterior glands within the parasitic platyhelminths (Neodermata). Future studies, however, to assess phylogenetic relationships within the Monogenea using anterior characters should prove informative.

1. INTRODUCTION

Attachment to a host is fundamental to the survival of many parasites. Parasites may invest considerable time and energy in reproducing themselves, in locating and then infecting their host organisms, which are often specific species or small groups of species (= host-specificity; e.g. Noble *et al.*, 1989; Rohde,

1993, 1994a). When their host has been located and/or infected, most parasites must attach themselves immediately at the site of contact where they may remain or they may migrate to a new site (e.g. Sukhdeo and Bansemir, 1996). The maintenance of firm attachment, often in or on a specific site (= site-specificity; e.g. Rohde, 1993, 1994a; Sukhdeo and Bansemir, 1996), is essential for the successful establishment of many parasites which may then proceed to feed, grow, mature and reproduce in or on the definitive host or hosts. Several parasite groups are recognizable instantly by the type of prominent organs that attach them mechanically and securely either inside or outside their host(s). For instance, parasitic copepods are often highly modified from their free-living counterparts and some have developed modified antennae (e.g. ergasilids), modified cephalothorax and thorax (e.g. lernaeids and pennellids) or a new organ, the bulla, formed from head and maxillary gland secretions (e.g. lernaeopodids) (Roberts and Janovy, 1996). Despite their conserved body plan, diversity is also known in attachment organs among some parasitic nematodes.

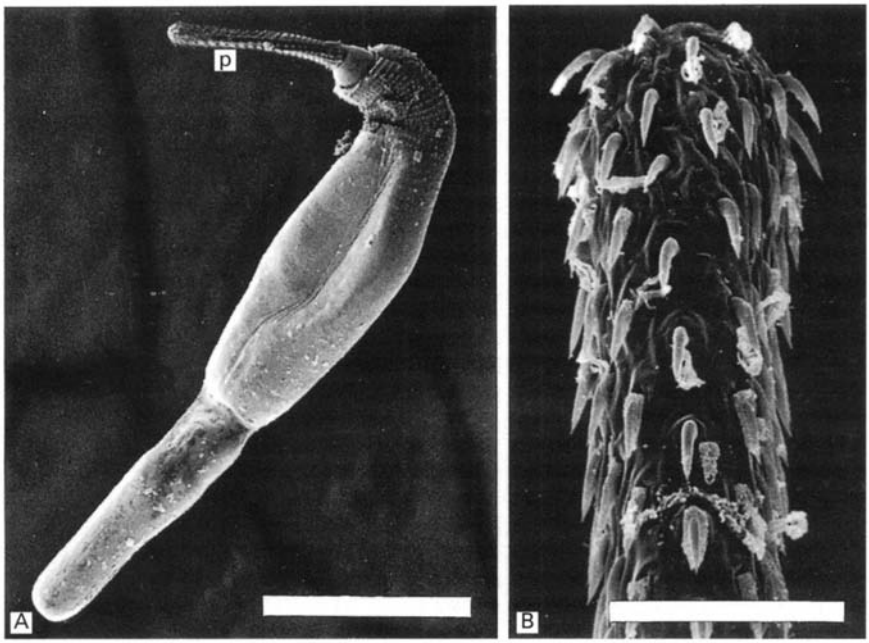


Figure 1 Scanning electron micrograph of *Telosentis australiensis* (Acanthocephala) from the gut of the eel *Anguilla reinhardtii* (Anguillidae) from south-east Queensland, Australia. A. Whole parasite showing anterior proboscis (p). Scale bar = 1 mm. B. Anterior proboscis adorned with hooks for attachment. Scale bar = 100 μm. (I.D. Whittington and B.W. Cribb, original photographs.)

For example, hookworms and strongyles (Strongylida) have conspicuous buccal capsules for attachment to the lining of the gastrointestinal tract of the host (Anderson, 1992). The morphology and evolution of cephalic structures within the Trichostrongyloidea were reviewed by Durette-Desset (1985). Acanthocephalans, or the thorny-headed worms, possess a fluid-filled proboscis usually adorned with hooks (Figure 1) for attachment to (Hayunga, 1991), or complete perforation of, the gut wall.

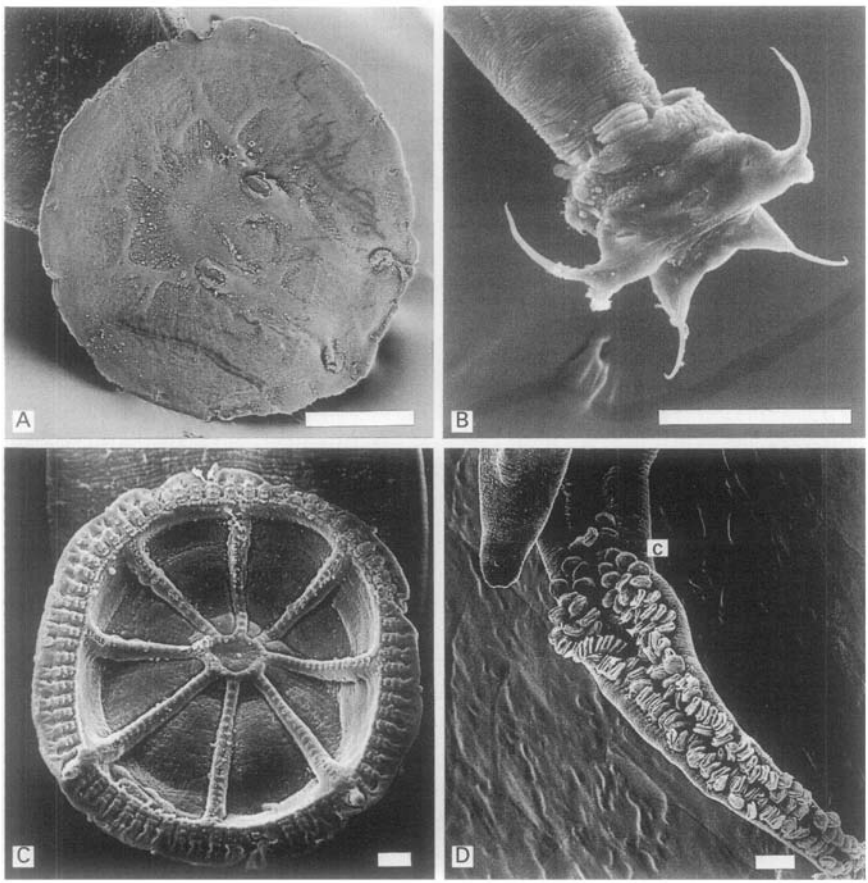


Figure 2 Scanning electron micrographs of the haptors (= posterior attachment organs) of some monogenean parasites. A. *Entobdella australis* (Monopisthocotylea: Capsalidae). B. *Chauhanellus australis* (Monopisthocotylea: Dactylogyridae). C. *Monocotyle spiremae* (Monopisthocotylea: Monocotylidae). D. Clamps (c) of *Gotocotyla* sp. (Polyopisthocotylea: Gotocotylidae). Scale bars = 100 μm. (B.W. Cribb and I.D. Whittington, original photographs.)

This review focuses on the Platyhelminthes (the flatworms). Each of the three major groups of parasitic platyhelminths also possesses its own highly characteristic organs for mechanical attachment to its hosts. Monogeneans, principally ectoparasites of fish, have a posterior attachment organ called the haptor equipped with hooklets, hooks, suckers, clamps and cement or combinations of these (Whittington and Combes, 1994; Figure 2). Cestodes include the endoparasitic true tapeworms (Eucestoda), the amphilinideans and the gyrocotylideans (see Rohde, 1994b). Most eucestodes have an anterior scolex provided with suckers of different kinds and may bear hooks at the apex of the worm, on the suckers themselves or on separate tentacles (e.g. trypanorhynchs; Figure 3A). Digeneans, the largely endoparasitic flukes, have acetabula or suckers and typically these are arranged as an oral sucker that leads to the gut and a ventral sucker which ends blindly (Figure 3B). The haptor of monogeneans (e.g. Llewellyn, 1957, 1958; Kearn, 1964, 1971a; Roubal and Whittington, 1990; Kearn and Bijukumar, 1997; Chisholm and Whittington, 1998a), the scolex of tapeworms (e.g. McVicar, 1972; Coil, 1991) and the suckers of digeneans (e.g. Halton, 1967; Smyth and Halton, 1983) have received some attention in terms of their structure and functional morphology. The Platyhelminthes also includes the largely free-living turbellarians although permanent symbiotic associations between turbellarians and other organisms are well known (Jennings, 1997; Rohde, 1997). While the majority of free-living turbellarians are tiny and occupy interstitial spaces in the meiofauna, some are large and conspicuous, such as the triclads (planarians) and marine polyclads. A special feature of tiny turbellarians is their ability to produce secretions (adhesives) for temporary attachment to sand grains and other small particles in their interstitial environments (Tyler, 1976). The adhesive secretions of symbiotic turbellarians such as some rhabdocoels and the temnocephalans may have evolved further to help attach them to their hosts (Jondelius, 1992; Kearn, 1998), but these adhesives have received less attention than those of the free-living interstitial taxa (Sewell and Whittington, 1995).

Despite the presence of a conspicuous and efficient haptor in monogeneans, the additional occurrence of anterior secretions with an adhesive role has been known for some time. There have been relatively few studies, however, that have investigated the nature and properties of these sticky secretions (Kritsky, 1978; El-Naggar and Kearn, 1980, 1983; Rees and Kearn, 1984; Cribb *et al.*, 1997, 1998; Kearn and Evans-Gowing, 1998; Whittington and Cribb, 1998a, b, 1999). This dearth of knowledge is surprising because monogenean adhesives display exceptional properties. They can adhere instantly and firmly to host epithelial tissues, surfaces that are inhospitable and 'designed' to prevent attachment (Ebran *et al.*, 1999). Furthermore, the strong adhesive bond can be severed equally instantly and is controlled precisely by the parasite. Apart from the obvious curiosity that such adhesive secretions evoke about their mechanism of operation, knowledge and understanding of their nature and

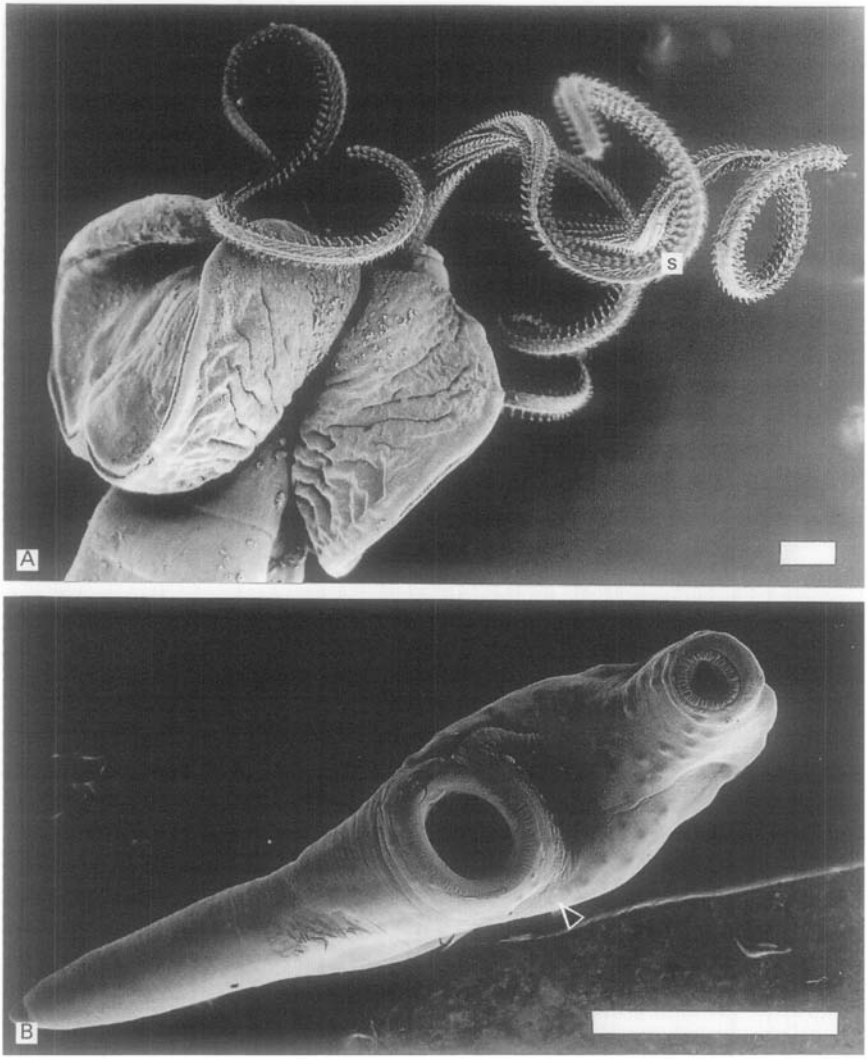


Figure 3 Scanning electron micrographs of the principal attachment organs of tapeworms and digeneans. A. Anterior scolex displaying spiny tentacles (s) of a metacestode, *Floriceps minacanthus* (Cestoda: Trypanorhyncha), removed from a cyst from the flesh of coral trout, *Plectropomus leopardus* (Serranidae) caught at Heron Island, Queensland, Australia. Scale bar = 100 μm. (B.W. Cribb and I.D. Whittington, original photograph; specimen identified by Dr M.K. Jones). B. Oral and ventral (arrowhead) suckers of *Hysterolecithoides frontilatus* (Digenea: Hemiuridae) from *Siganus nebulosus* (Siganidae) collected at Noosa, Queensland, Australia. Scale bar = 1 mm. (B.W. Cribb and I.D. Whittington, original photograph from a specimen provided and identified by Dr T.H. Cribb.)

interactions after further research may lead to the development of adhesives of potential commercial use. Furthermore, information about how the adhesives work may lead to a method to prevent their operation in nature as a possible control measure for pathogenic monogeneans in aquaculture.

Our review focuses on adhesives, in the sense of attachment by chemical means, using a thin layer of adhesive material (Section 2.1) between a symbiotic or parasitic metazoan (here, we concentrate on platyhelminths) and its host. We are aware that adhesion among bacteria and protists is mediated by a variety of surface molecules such as lipophosphoglycans (Sacks *et al.*, 1994), lectins (Ortega Barria and Boothroyd, 1999) and proteins (Silva-Filho *et al.*, 1988; Coppel *et al.*, 1998; Alderete, 1999; Ghosh *et al.*, 1999; Kennett *et al.*, 1999; Yuda *et al.*, 1999), which can act as 'adhesins' for recognition and binding to host cells as a preparatory step for infection (Whittington *et al.*, 2000a). Molecular characterization of adhesins is receiving close attention (e.g. Alderete, 1999; Kennett *et al.*, 1999). Further study may demonstrate whether there are parallels among the molecular interactions between the surfaces of prokaryotes, single-celled eukaryotes and their host cells via adhesins and the types of bioadhesives secreted by metazoan organisms that attach to living surfaces. Bioadhesion by organisms to epithelial surfaces of other organisms, however, has received little attention thus far.

The concept for this review has grown from our escalating interest in the structure and morphology of the anterior adhesive areas of monogeneans and the form and ultrastructure of their secretions (Cribb *et al.*, 1997, 1998; Whittington and Cribb, 1998a, b, 1999; Whittington *et al.*, 2000a). We recognize that their remarkable properties raise several fundamental questions. How is adhesion to a living surface generated so rapidly? What causes instant detachment? What is the chemical nature of the secretions? Could the chemistry of the adhesive play a role in parasitism by monogeneans and help determine their strong host- and site-specificity? How similar or different are the adhesive secretions across the Monogenea? Evolutionary implications within and among the flatworms also emerge. Adhesives are known widely among free-living and some symbiotic turbellarians and in the ectoparasitic monogeneans, but, at first sight, there appears to be significantly less reliance on adhesive secretions among the largely endoparasitic Cestoda and Digenea. It is possible, however, that the use of adhesion in these endoparasitic platyhelminths has been overlooked (Kearn, 1998). At different times in their life cycles, various stages of cestodes and digeneans are known to possess conspicuous glands, often located anteriorly. The scope of this review therefore encompasses some of what is known about these other anterior glands and their secretions. There has been no previous review of adhesion and adhesives across the Platyhelminthes and we seek to redress this. Attachment by adhesion is known elsewhere in the invertebrates (e.g. in molluscs, crustaceans and echinoderms) and this raises broader questions about similarities and

differences between the adhesives evolved by different animals to solve similar problems for firm, but often temporary, attachment to a variety of different substrates.

In this review, we aim to address the questions evoked above by summarizing knowledge on adhesion in general and then we focus specifically on adhesive systems in biology that have relevance to the remarkable temporary adhesion in turbellarian and monogenean flatworms. The diversity of anterior glands in larval, metacestode and adult cestodes and in the miracidia, cercariae and adults of digeneans also receive attention. It is our intention to consolidate appropriate information on the adhesives of platyhelminths to help pave the way for future studies that will attempt to address, in particular, deficiencies in our understanding of temporary adhesion to live, inhospitable surfaces. This aspect of the parasite–host interface has been neglected previously.

2. ATTACHMENT BY ADHESION

As a method of attachment in nature, adhesion is ubiquitous. It ranges from attachment by bacteria to various supports and films (Walker, 1987; Characklis, 1990) to firm anchorage by large metazoans such as tree frogs to diverse substrates such as leaves, bark and glass (Nachtigall, 1974). As with some other modes of attachment that have been plundered from nature (Whittington and Cribb, 1998b), the development of glues from natural adhesives is a lucrative business and an expanding area of science, not just to produce new adhesives but also to prevent biofouling (Clare *et al.*, 1998). Indeed bioadhesion is now a recognized subdiscipline of biology and the journal *Biofouling* (published by Harwood Academic Publishers) is subtitled The Journal of Bioadhesion and Biofilm Research. Burzio *et al.* (1997) commented that biological adhesives are 'inspiring to a world hungry for new adhesives and their potential applications'. This is certainly reflected by the diversity of projects and studies on bioadhesives. For example the following studies (and this is not an exhaustive list) have investigated adhesive proteins of various species of mussels: Waite *et al.* (1989); Williams *et al.* (1989); Strausberg and Link (1990); Rzepecki *et al.* (1991); Saez *et al.* (1991); Rzepecki and Waite (1995); Schnurrer and Lehr (1996); Deacon *et al.* (1998) and Takeuchi *et al.* (1999). In addition to mussels, other candidate organisms that have received attention are anemones, limpets, tubeworms (Walker, 1987), annelids and tunicates (Rzepecki and Waite, 1995), barnacles, kelp and sea moulds (Burzio *et al.*, 1997). From this, it is clear that much focus has been directed to sessile marine organisms because their adhesion is extremely strong and resists powerful shear forces in their environments (e.g. ship hulls, rocky coastlines and jetty pylons; Schnurrer and Lehr, 1996).

In fact mussels are described to have 'an uncanny ability to stick to hard, wet surfaces' (Rzepecki *et al.*, 1991). There is, therefore, obvious interest in the biology, functional morphology, chemistry and potential use of adhesives from these organisms that attach permanently. Adhesion by another group of marine organisms, the echinoderms, has been the subject of recent research (review by Flammang, 1996, and references therein; Flammang and Walker, 1997; Flammang *et al.*, 1991, 1994, 1998), but it is adhesion of a different kind. Unlike the marine organisms noted above that are mostly sedentary and adhere permanently, echinoderms such as sea stars and sea cucumbers use temporary adhesion to benthic substrates and, as pointed out by Flammang *et al.* (1998), mechanisms for temporary adhesion are less well understood than those for permanent adhesion.

2.1. Physics of Adhesion

Adhesion is the word used in general terms to describe attachment by chemical means achieved through adhesive secretions (Nachtigall, 1974; Flammang, 1996). An alternative definition by Wake (1987) and Walker (1987) is the chemical attraction between substances that requires 'work' to separate them after they are brought into contact. Walker (1987) emphasized that the word adhesion should be restricted to cases where the surfaces or substances that contact each other, and which may then separate, maintain their identity and integrity throughout the process. If two inert objects were to be glued together using commercial epoxy glue, each surface usually has glue applied to it. The glue adheres or holds to each surface by *adhesion* and can be described as the intermolecular forces between two dissimilar materials (Figure 4A). When each of the surfaces that is coated with the tacky glue are pressed together, the two coatings of glue merge by *cohesion*, which can be described as the intermolecular forces between identical materials (Figure 4B). In nature, cases of cohesion are scarce because circumstances whereby two surfaces are similarly precoated by a glue are rare (Nachtigall, 1974). Adhesion, however, is much more common because organisms can secrete glues from glands as and when they prepare to attach themselves to a substrate that is not similarly treated (Figure 4C). Nachtigall (1974) commented that despite the comparative prevalence of adhesion in biology, there was little knowledge of the molecular mechanisms involved. It is this topic that has matured into the discipline of bioadhesion and the continual advance of biochemical and molecular technologies has revealed significant knowledge about adhesion by some organisms that have become favoured model systems (e.g. mussels, limpets, barnacles and echinoderms; see Section 2 above). For more detail on the diversity of methods of attachment by adhesives in biology, the reader is referred to Nachtigall (1974).

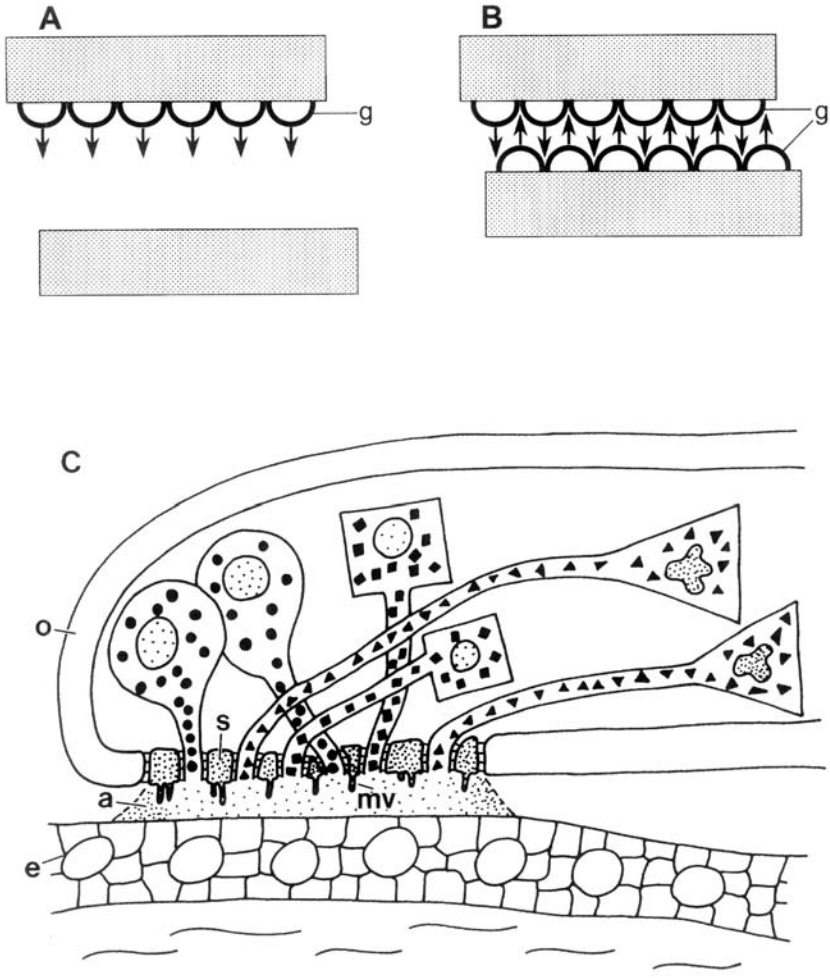


Figure 4 The physical mechanisms of adhesion. A. Adhesion involves intermolecular forces between two dissimilar materials. Here, the 'glue' (g) interacts with each surface by adhesion. B. Cohesion involves intermolecular forces between identical materials. In this case, two coatings of glue (g) merge by cohesion. (A and B redrawn and modified from Nachtigall, 1974). C. Diagrammatic representation of *tissue adhesion* (see Section 3.4) depicting an organism (o) that can release up to three different secretory types (circles, squares and triangles) from three different gland cell types (circular, square and triangular) to adhere to an epithelial surface (e). This diagram is based on knowledge from studies of the anterior adhesive areas of monopisthocotylean monogeneans and their temporary attachment to host epithelium (Section 5.2.2). Note the layer of adhesive material (a), the specialized adhesive area tegument (s) and associated microvilli (mv). (B.W. Cribb and I.D. Whittington, original drawing.)

3. TYPES OF ADHESION

In biology, three types of adhesion have been identified and distinguished previously, but each serves different purposes (Tyler, 1988; Flammang, 1996; Flammang *et al.*, 1998) and, as will be described later (Sections 9 and 10), each adhesion type may use different adhesive systems. Here, we introduce a fourth type of adhesion that concerns *temporary* but firm attachment using adhesives to a *living* surface (Section 3.4) rather than to an inert, abiotic substrate (Section 3.3).

3.1. Permanent Adhesion

Permanent adhesion concerns the release of cement for permanent attachment of organisms that are sessile and remain on a single abiotic substrate throughout their life after initial settlement, usually as a larva. Substrates that these organisms attach to are usually subject to powerful shear forces, commonly tidal action, and examples include attachment by microalgae, macroalgae, mussels, barnacles and tubeworms to rocks and the hulls of ships (e.g. Walker, 1987; Tyler, 1988; Schnurrer and Lehr, 1996; Burzio *et al.*, 1997). Animals that display such permanent attachment are among the best studied for adhesion. Their adhesive system comprises a single type of secretory cell or, if more are present, only one cell type is usually involved in adhesion (Walker, 1970; Tamarin *et al.*, 1976; Waite, 1983; Walker, 1987).

3.2. Transitory Adhesion

This is characteristic of those invertebrates that move across a substrate by ciliary gliding (e.g. some turbellarians; see Martin, 1978; Rieger *et al.*, 1991) or muscular wave-like activity (e.g. foot secretions of gastropod molluscs; Grenon and Walker, 1978, 1980; Walker, 1987) and enables adhesion and movement along a substrate simultaneously. Attachment by limpets (*Patella*) is achieved by a thin film of mucus secreted from six types of secretory cells in the foot and this viscous material is left behind as they progress (Grenon and Walker, 1978; Walker, 1987). The pedal disc of sea anemones operates by a similar system (Flammang, 1996). For more details on transitory adhesion in molluscs and coelenterates, see reviews by Walker (1987) and Flammang (1996).

3.3. Temporary Adhesion

Temporary adhesion permits an organism to attach firmly but fleetingly to a substrate such as sand grains, other sediment, rocks and a variety of other abiotic surfaces. Momentary attachment by adhesives is displayed among tiny invertebrates that inhabit interstitial spaces, such as many groups of free-living turbellarians (Tyler, 1976, 1988; Rieger *et al.*, 1991) and gastrotrichs (Tyler and Rieger, 1980). It also includes the mechanism of adhesion identified for the podia (= tube feet) of echinoderms (Flammang, 1996). In most of these examples, the adhesive system comprises two kinds of gland cells that are associated closely and has been termed the 'duo-gland' system whereby one cell type produces the adhesive and the other cell type is thought to produce a secretion that causes detachment (Tyler, 1976). The duo-gland system will be dealt with in more detail in Sections 4.4 and 4.9. Temporary adhesion is also used by the cypris larvae of barnacles during the process of finding a suitable surface or platform on which to settle before they attach themselves permanently for metamorphosis into the shelled juvenile (Walker, 1987). A single secretion is thought to be responsible for their temporary adhesion (Walker, 1987). The fact that most marine invertebrate larvae have 'the power of choice' at settlement, whether temporary or permanent, is reviewed by Hadfield (1998) and is discussed in the context of habitat selection by Whittington *et al.* (2000a).

3.4. Tissue Adhesion: Temporary Adhesion to Living Surfaces

All of the above examples relate to the importance of adhesion for free-living organisms, mostly but not exclusively in a marine environment, to maintain secure attachment to inert, abiotic substrates. Walker (1987) states that any surface placed in sea water becomes coated rapidly by a monolayer of polymeric material comprising deposited or adsorbed macromolecules, most of which are proteins. Subsequently, bacteria attach to the 'conditioning film' and form the 'primary film' (Walker, 1987). The primary film is a moderately negatively-charged layer which coats all marine surfaces (Characklis, 1981; Walker, 1987; Flammang, 1996), but it is not a living layer. A large proportion of organisms, however, has adopted a parasitic lifestyle (Price, 1977; Windsor, 1998) and live in or on another living organism, the host. Unlike free-living organisms, parasites of invertebrates and vertebrates must contend with a concerted effort by the host's immune system to control or reduce their populations or to remove them completely. There are many examples of the subterfuges evolved by parasites that enable them to live in sites that are particularly immunologically active, such as the blood system (e.g. molecular mimicry; Behnke, 1990; Kearn, 1998). Symbiotic and parasitic flatworms

attach to a variety of cuticular, exoskeletal, epithelial and membranous surfaces in or on their hosts and their mechanical methods of attachment were referred to in the Introduction. The significance of adhesion to *living* tissues by parasites, however, is an area that has received no attention. Some barnacles are known to 'infect' whales, but there has been a surprising lack of study of this phenomenon although there are reports of tissue reactions against these invaders (Ridgway *et al.*, 1997). Here, we propose the term *tissue adhesion* as a fourth type of bioadhesion, the importance of which has not been recognized to date.

Relatively hard, rigid and non-secretory surfaces such as the carapace of crustaceans probably provide a relatively stable and non-threatening surface for attachment by platyhelminths known to use adhesives such as the temnocephalans (Sewell and Whittington, 1995). Attachment to cuticular surfaces probably falls between temporary adhesion and tissue adhesion. The biggest likely threat to temnocephalans is moulting when the external cuticle of the crustacean host is shed. Epithelial tissues that line the intestine of vertebrates (e.g. Castro and Harari, 1982; Ishikawa *et al.*, 1994) and which cover the body surfaces, scales, fins and gills of fishes (e.g. Buchmann, 1998a, 1999; Whittington *et al.*, 2000a) are active secretory layers that grow, regenerate and produce mucus containing peptides and carbohydrates that may have immunological activity. The mucus of fish, produced by the goblet or mucous cells in the epithelium, is known to be highly variable chemically between species and there are accumulating data for the presence of a variety of non-specific immune responses in the mucus of fish infected by monogenean parasites (Buchmann, 1998b, 1999; Whittington *et al.*, 2000a). Furthermore, specific antibodies and lymphocytes may also be involved (Buchmann, 1999). Therefore, epithelial surfaces of fishes and also of amphibians (Whittington *et al.*, 2000a) must be considered highly inhospitable and their physical properties (i.e. slimy and wet) appear to be a deterrent to colonization by organisms (Ebran *et al.*, 1999) and 'designed' to prevent adhesion. Nevertheless, adhesion to such surfaces, especially by many species of monogeneans, is achieved with considerable success. That there may be chemical differences between the mucus of different species of fish (Buchmann, 1998a) or even between mucus from different sites on the same species of fish (Buchmann and Bresciani, 1998) may have implications for the host- and site-specificity of monogeneans. This is considered further by Whittington *et al.* (2000a) and in Section 5.7. If mucus or perhaps some specific chemical signal in mucus or from epithelial tissue of fishes is involved in monogenean host- and/or site-specificity, it has interesting parallels with the apparent favourability or unfavourability of inert substrates in the marine environment that can be detected, identified and 'chosen' before settlement by the pelagic larvae of many free-living, benthic marine invertebrates (Hadfield, 1998).

3.5. Summary

Crisp (1974) first recognized that biological adhesives could be divided into two groups, permanent and temporary. Current knowledge has added a further category, transitory adhesion, as outlined above (Tyler, 1988; Flammang, 1996) and we have added tissue adhesion, the temporary adhesion using bioadhesives to live surfaces, the importance of which will be advanced further in Sections 5.7, 9 and 10. In a summary of adhesion by marine organisms, Walker (1987) listed four main theories for mechanisms that generate permanent adhesion: diffusion; mechanical interlocking; electrostatic; and adsorption. The latter at the time was the most widely accepted theory of permanent adhesion. In the quarter century since the excellent synopsis of Nachtigall (1974), various hypotheses and molecular models have been proposed to explain temporary adhesion, such as the so-called duo-gland adhesive system (Tyler, 1976; Hermans, 1983), which according to Walker (1987) was speculation with no real evidence. Current interpretations of adhesion theory for various biological adhesives and suggested functional mechanisms are reviewed in Section 9.

4. ATTACHMENT BY ADHESIVES IN THE TURBELLARIA

Turbellarians include free-living representatives such as the acoels, rhabdocoels, triclads and polyclads as well as some groups that can form associations or relationships with invertebrates and vertebrates, such as some species of polyclads and triclads, the temnocephalans, umagillid and graffillid rhabdocoels and the fecampiids (Kearn, 1998). The name 'Turbellaria' refers to a group of flatworms that are now known to be paraphyletic (Rieger *et al.*, 1991; Rohde, 1994b; Whittington, 1997) and in a strict sense, use of the word Turbellaria should discontinue. We, however, like others (e.g. Rieger *et al.*, 1991; Rohde, 1994b; Whittington, 1997) use the term here for convenience. It is unfortunate that the names of many of the higher taxa which specialists of the turbellarians use can be confusing, even to scientists familiar with the Platyhelminthes, and groupings can have different meanings when used by different turbellarian authorities. In an attempt to clarify some of these groupings of taxa, whether clades, subclades, orders or suborders, we refer the reader to Table 1 in Rieger *et al.* (1991). However, due to the dynamism of the classification system of turbellarians, some taxonomic affiliations may have changed since the reports reviewed here were published and, for clarity, we have left most taxa unchanged from primary sources. In addition to a relatively recent summary of turbellarian systematics, Rieger *et al.* (1991) provide an excellent overview of the general structure and anatomy of turbellarians.

Free-living turbellarians can inhabit fresh water, marine and terrestrial environments. Like numerous other animals that may live in the marine interstices, small turbellarians have the ability to attach themselves temporarily to surfaces using special adhesive structures. Turbellarians have a variety of epidermal and subepidermal gland cells that manufacture a range of secretions that are discharged to the surface of the worms. These secretions are considered to play an important role in turbellarian biology and in addition to attachment to, and detachment from, a substrate, they may function in gliding motion, protection from predators and prey capture (Cannon and Watson, 1996). For this review, we address accumulated knowledge on sticky secretions or viscid substances produced by the gland cells of a range of free-living and symbiotic turbellarians. Unlike the Monogenea, cestodes and the Digenea reviewed later, the Turbellaria are well known for their adhesive secretions and there is a substantial body of knowledge about the group. Mostly, this is from comparative ultrastructural studies used to supplement and extend the relatively small numbers of other available characters present in these flatworms for phylogenetic purposes (Tyler, 1976). These studies, therefore, have been employed in much the same way that sperm morphology (e.g. Justine, 1991), flame bulb ultrastructure (e.g. Rohde, 1990) and now molecular data (e.g. Carranza *et al.*, 1997; Littlewood *et al.*, 1999; Litvaitis and Rohde, 1999) have been used for the entire Platyhelminthes. The ultrastructural studies by Tyler and colleagues (e.g. Tyler, 1976, 1988; Smith *et al.*, 1982) and some histochemical analyses (e.g. Smith *et al.*, 1982; Tyler, 1988) have also contributed to a functional model that attempts to explain how adhesion may be generated in some turbellarians (e.g. Tyler, 1988).

4.1. Use of Adhesives by Turbellarians

Many tiny invertebrates that live in the interstitial environment can adhere temporarily to a substrate, e.g. Turbellaria (see Tyler, 1976); Gastrotricha (see Tyler and Rieger, 1980); Nematoda (see Adams and Tyler, 1980); and Polychaeta (see Gelder and Tyler, 1986). Tyler (1976) describes that when a dish is shaken containing free-living turbellarians from coastal sand samples, the worms stick tenaciously to the bottom of the vessel; a similar behaviour occurs when subjected to jets of water from a pipette. His observations show that the turbellarians flatten their bodies against the bottom of the dish and apply their adhesive organs to the vessel surface, using more adhesive organs when the strength of the water current increases. *Haplopharynx* (Archoophora: Haplopharyngida) was seen to use its posterior organs most frequently for attachment, but progressively used its more anterior organs if sufficiently disturbed such that attachment could occur at several places along the body (Tyler, 1976).

The adhesive organs of interstitial turbellarians can also be used to reduce

the speed of, or halt, forward swimming by their application to a substrate (Tyler, 1976). *Prosorhynchus* (Neoophora: Lecithoepitheliata), a non-interstitial freshwater species, employs its adhesive organ in this manner as also do Proseriata which are rapid swimmers (Tyler, 1976).

Movement along a substrate can also occur, especially when retreating from an obstacle or noxious entity. Tyler (1976) notes that inchworm movement can occur in reverse, especially in the otoplanids which 'can use their posterior adhesive organs to virtually hop backwards, attaching and reattaching their tail tip as rapidly as five or six times a second'.

In the Temnocephalida, most of which are ectosymbionts of crustaceans, adhesives are used for feeding (Jennings, 1968), attachment and locomotion (Sewell and Whittington, 1995). Some turbellarians parasitize fishes and the triclad, *Micropharynx parasitica,* attaches to the dorsal body surface of its elasmobranch ray host using a posterior adhesive pad (Ball and Khan, 1976) and has been mistaken as a monogenean even by specialists (Kearn, 1998). Indeed the presence of attachment devices in extant free-living turbellarians and the fact that similar organs were likely in ancestral turbellarians has prompted Kearn (1998) to suggest that they represented important preadaptations in a progression, among some lineages, to symbiosis. Turbellarian adhesives are also used for other purposes such as feeding, already mentioned for temnocephalans, and a report by Jones and Cumming (1998) describes fishing for termites outside a termite mound by the planarian *Microplana termitophaga*, using its adhesive front end.

Many studies on the ultrastructure and comparative morphology of the adhesive systems of turbellarians have focused on free-living representatives (see Tyler, 1976, 1988). Adhesive glands of the entosymbiotic Pterastericolidae from the digestive tract of starfish were reviewed by Jondelius (1992) and there have been several studies of gland cells that may have an adhesive function in the Temnocephalida (see Williams, 1994; Joffe *et al.*, 1995a, b; Rohde and Watson, 1995; Sewell and Whittington, 1995; Sewell, 1997). There is much literature on turbellarian secretions and we have been selective to convey generalities among their adhesive systems.

4.2. The Array of Gland Cells and Secretory Products in Turbellarians

There is a bewildering assemblage of epidermal and subepidermal gland types among turbellarians, but little is known of the composition or function of most of them (Rieger *et al.*, 1991). Most glands are unicellular. The gland cell bodies lie in the parenchyma and ducts (usually termed 'necks' in the turbellarian literature because they stand proud of the surrounding epidermis to form a papilla) open between or through epidermal cells (Figure 5). A single

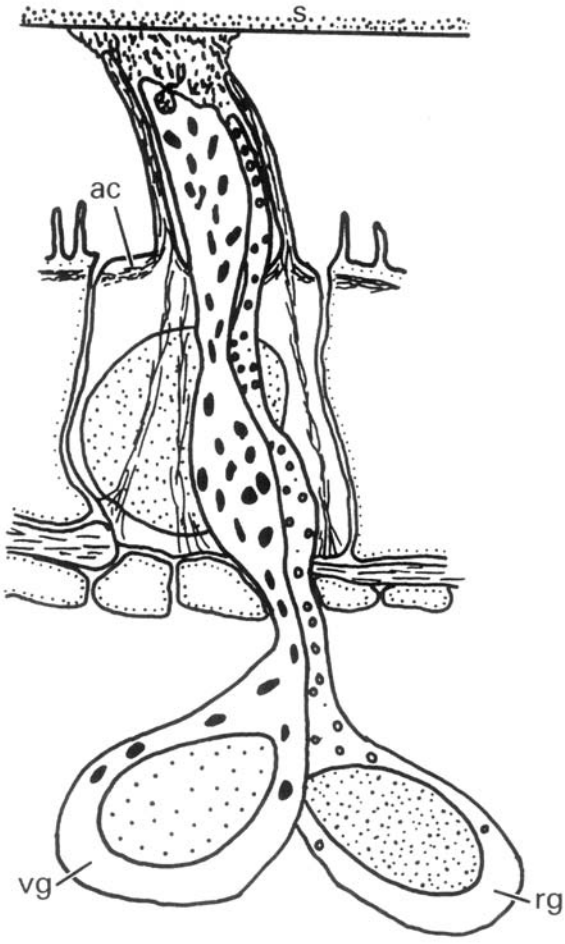


Figure 5 Diagram of typical duo-gland system of turbellarians showing anchor cell (ac), viscid gland (vg), releasing gland (rg) and substrate (s). (Redrawn from Tyler, 1988.)

layer of microtubules usually lines the neck (Rieger *et al.*, 1991 and Figure 6). Based on light microscopy, Hyman (1951, p. 72) grouped the glands as either cyanophilous (mucous-producing) or eosinophilous (adhesive). Glands with necks that emerge at a variety of sites over the surface of the body probably produce a protective coating of slime or similar material whereas when necks are located mostly ventrally, the slime secreted may be for locomotion (Rieger *et al.*, 1991). A rod shape is an especially common form among turbellarian secretory bodies (Section 4.3). For an overview and associated references, see Rieger *et al.* (1991). With such a diversity of secretions and gland cells among the turbellarians, it is hardly surprising that so few have been characterized.

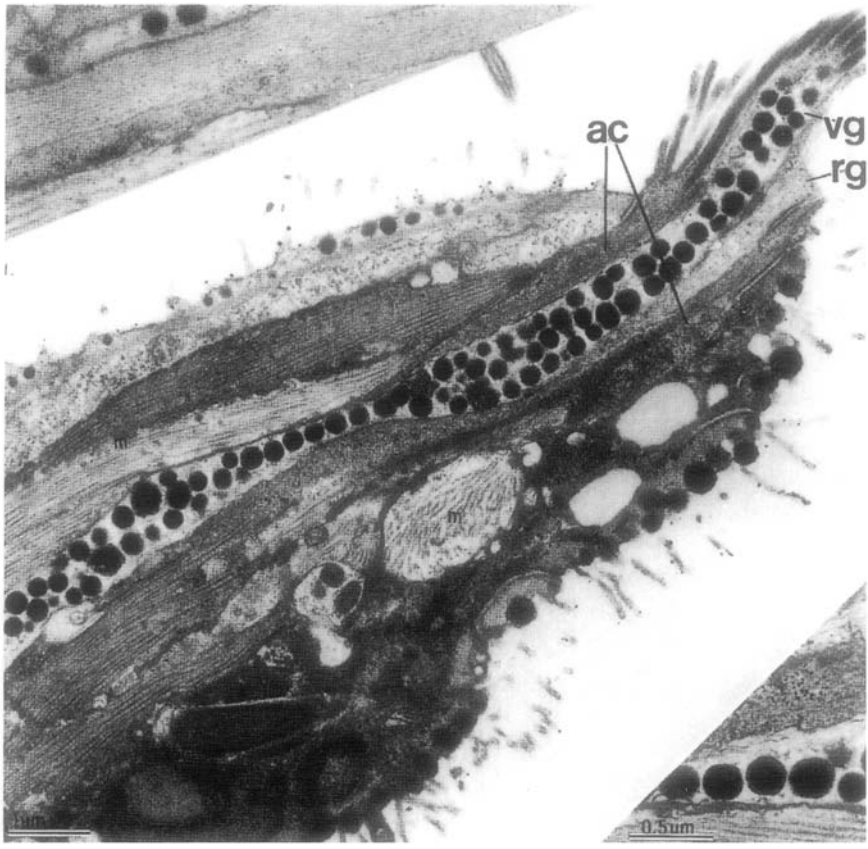


Figure 6 Transmission electron micrographs of the tail tip of the turbellarian *Psammomacrostomum* sp. (Archoophora: Macrostomida) in longitudinal section showing an anchor cell (ac), viscid gland granules (vg) and releasing gland granules (rg). Scale bar = 1 μm. Upper inset shows microtubules in anchor cell 'necks'. Lower inset shows granules in viscid gland 'neck'. Both insets are to the same scale; scale bar = 0.5 μm. (Reproduced with permission from Tyler, S. (1976). Comparative ultrastructure of adhesive systems in the Turbellaria. *Zoomorphologie* **84**, 1–76. Springer-Verlag, Berlin, Heidelberg.)

4.3. 'Rhabdoid' Secretions

Electron microscopy has enabled comparative studies of the ultrastructure of some of the adhesive systems from a variety of turbellarians (e.g. Tyler, 1976, 1988; Ehlers, 1992; Jondelius, 1992; Sections 4.4, 4.5 and 4.6). It has also been used to compare the subcellular structure of so-called 'rhabdiform' secretory bodies (Smith *et al.*, 1982). Rhabdiform describes a rod shape, usually with

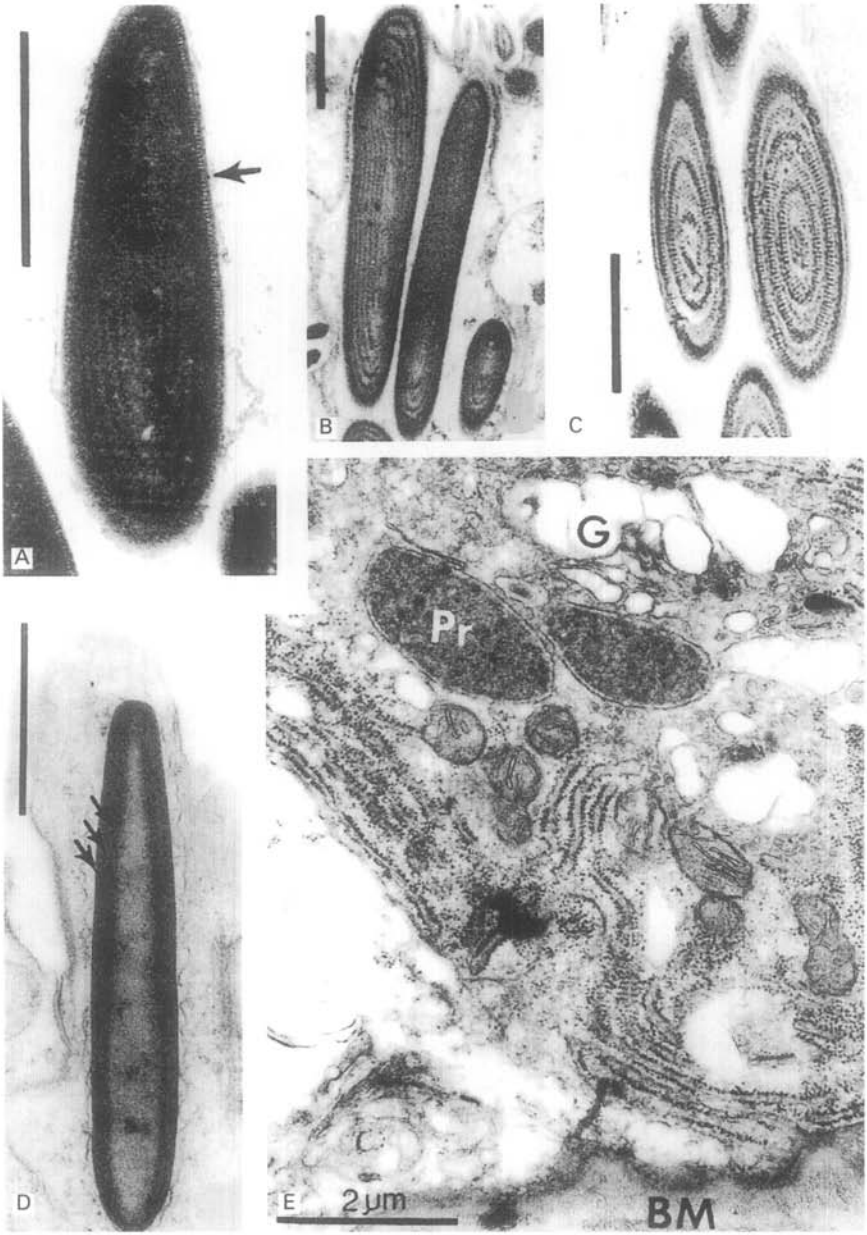


Figure 7 Transmission electron micrographs of turbellarian rhabdites. A. Oblique section of mature rhabdite from *Microstomum* sp. (Macrostomida) showing lamellate structure and radial striations of outer sheath (arrow). B. Longitudinal section of rhabdites in gland cell 'neck' from *Myozona* sp. (Macrostomida). C. Oblique section of mature rhabdites from

bluntly rounded ends and it may be slightly bowed (Figure 7). Rhabdiform secretions are particularly common and a variety of gland cells in turbellarians may produce them. We review these before providing an overview of some of the adhesive systems recorded in the group.

Rhabdoid is the general term used for rhabdiform secretory products among turbellarians and embraces the following secretory granules: rhabdites, rhammites, some epitheliosomes and various other, as yet unnamed and uncharacterized, secretory products (Rieger *et al.*, 1991). It was Hyman (1951) who first defined the term rhabdite as those rhabdoid secretions of turbellarians that are shorter than the height of the epidermis and are produced and secreted by glands located either in the epidermis (termed epidermal rhabdites) or beneath the epidermis in the mesenchyme (termed adenal rhabdites). Rieger *et al.* (1991) emphasized that prior to the recognition of their special nature, the term 'rhabdite' was applied indiscriminately to a range of rhabdoids that now fall outside the refined and tight definition and some have applied the term rhabdite even further afield among other invertebrate groups (Smith *et al.*, 1982). Rhabdites are rod-shaped, epidermal, mucous-secretion bodies found in most turbellarians and previous studies have demonstrated their unusual and highly characteristic ultrastructure (e.g. Reisinger and Kelbetz, 1964; Lentz, 1967; Reisinger, 1969; Figure 7). Rhabdite glands are common over most of the body surface but are usually more numerous dorsally or may be grouped at particular, special sites such as the mouth, female genital pore and anteriorly among other glands comprising the frontal glands (Section 4.5). Since rod-shaped bodies are common in the anterior secretions from some adhesive gland cells in monopisthocotylean monogeneans (Section 5.2), an account of knowledge of rhabdites and other rhabdoid secretions in turbellarians is considered especially desirable here.

A study of the ultrastructure and histochemistry of mature rhabdites and their formation in 14 species of turbellarians from the orders Acoela, Macrostomida, Rhabdocoela and Polycladida and comparison with details known of 'rhabdites' from other studies led Smith *et al.* (1982) to redefine the rhabdite and they identified three morphological variants. The revised definition, restricted to include only those rod-shaped bodies considered likely to be homologous (see also Figure 7) by Smith *et al.* (1982), is as follows:

Paramyozonaria sp. (Macrostomida) showing radial striations in each of the lamellae. D. Trilaminar cortex (three arrows) surrounding a heterogeneous core of a mature rhabdite from *Macrostomum* sp. 2 (Macrostomida) of Smith *et al.* (1982). All scale bars = 1 μm. E. Rhabdite gland cell from *Stylochus zebra* (Polycladida) lying just above the basement membrane (BM) within the epidermis. Protorhabdites (Pr) occur adjacent to a prominent Golgi apparatus (G) and are not surrounded by microtubules. Scale bar = 2 μm. (All photographs reproduced with permission from Smith, J.P.S. *et al.* (1982). The morphology of turbellarian rhabdites: phylogenetic implications. *Transactions of the American Microscopical Society* **101**, 209–228.)

> Rod-shaped secretions, of varying lengths and approximately 1 μm in diameter, which are acidophilic, refractile, and membrane-bounded, with one to several concentric striated lamellae constituting its cortex, and with a concentrically lamellated, granulated, or homogeneous medulla; formation within a gland cell with the cortical organization emerging first and with a microtubular sheath occurring external to the unit membrane; release to the exterior through neck(s) of the gland cells that protrude(s) either between epidermal cells or through epidermal cells.

The three morphological variants, although not necessarily functionally different, within this definition and identified by Smith *et al.* (1982) are: (i) multilamellated rhabdites (Figures 7A, B, C) that consist of a variable number of concentric lamellae and are found in members of the Macrostomida, Polycladida, Rhadocoela, Temnocephalida and in at least one member of the Proseriata ; (ii) triclad-type rhabdites that have only one fibrillar cortical layer and are found only in Tricladida (not shown); (iii) *Macrostomum*-type rhabdites that have three electron-dense fibrillar cortical layers and are found only in the genus *Macrostomum* (Figure 7D). These distinctions were used by Smith *et al.* (1982) to infer some phylogenetic conclusions (Section 4.9). Possession of lamellar rhabdites following the definition of Smith *et al.* (1982) led to the choice of the name 'Rhaditophora' by Ehlers (1985) for higher Turbellaria exclusive of Acoelomorpha and Catenulida (see Rohde, 1990; Rieger *et al.*, 1991). It is of interest to note that the presence of microtubules around forming rhabdites (although these are not present in specimens of the polyclad, *Stylochus zebra* (see Smith *et al.*, 1982 and Figure 7E)) is also a feature of forming rhabdiform secretory bodies in some larval and adult monopisthocotylean monogeneans (Section 5.2). Microtubules also occur inside secretory bodies of some larval amphilinids (Section 6.4).

In triclads, epitheliosomes can be mistaken for rhabdites. Rieger *et al.* (1991) consider that most references to rhabdites in triclad literature really pertain to rod-shaped epitheliosomes. However, as the detailed definition of a rhabdite above emphasizes, a critical and distinctive feature of them is their lamellate cortex (Figures 7A–D) which epitheliosomes lack.

Rhabdite function is generally considered to be for the formation of mucus used by some turbellarians for ciliary gliding (Smith *et al.*, 1982) and by others for prey capture, protection perhaps via predator repulsion, cocoon formation and excretion of metabolic wastes (Rieger *et al.*, 1991; Cannon and Watson, 1996). The protective role of rhabdites is extended considerably in *Notodactylus handschini* (Temnocephalida) because this species produces permanent surface structures in the form of dorsal scales derived from dorsally discharged rhabdites (Jennings *et al.*, 1992). A study by Sewell and Whittington (1995) on attachment and locomotion by the temnocephalan, *Craspdella pedum*, implied that tentacular rhabdites, of the

lamellate-type defined above by Smith *et al.* (1982), have a role in adhesion (Section 4.6).

Characteristically, rhammites at up to 30 μm long are far more elongate than rhabdites, most of which range in length from 3 to 9 μm (Rieger *et al.*, 1991). Furthermore, the distribution of rhammite glands is more localized and these are found commonly among the frontal glands at the anterior extremity of the body (see Rieger *et al.*, 1991 and Section 4.5). Rhammite glands can be prominent in representatives of the Macrostomida and some Rhabdocoela (see Rieger *et al.*, 1991). The ultrastructure of rhammites from the Macrostomida displays a dense cortex and homogeneous to granular medulla. Klauser and Tyler (1987) confusingly considered that these bodies could be a kind of rhabdite.

Other rhabdoid secretions are known in the lower turbellarians (Rieger *et* al., 1991): in acoels, these stain cytochemically as mucins and, in some catenulids, they can have a longitudinally striate structure. Other rhabdiform secretions among turbellarians include granules called chondrocysts and pseudorhabdites in terrestrial triclads, and sagittocysts among acoelomorphs (Rieger *et al.*, 1991).

4.4. Free-living Turbellarians with a Duo-gland System

Comparative ultrastructural studies summarized by Tyler (1976) have demonstrated that the adhesive systems of the following rhabditophoran turbellarian orders are composed of three distinctive cell types comprising two gland cell types and one non-glandular cell type (Figures 5, 6): Haplopharyngida; Macrostomida; Polycladida; Rhabdocoela (the Rhabdocoela 'Typhloplanoida' and Rhabdocoela Kalyptorhynchia of Ehlers and Sopott-Ehlers, 1993); Proseriata; marine Tricladida. Tyler (1988) added the freshwater Lecithoepitheliata and a species of *Microdalyellia* (Dalyelliidae), a freshwater dalyellioid rhabdocoel (Rhabdocoela 'Dalyellioida' of Ehlers and Sopott-Ehlers, 1993), to these taxa (see also Ehlers, 1989; Ehlers and Sopott-Ehlers, 1993).

Tyler (1976) explains how difficult it is to resolve the functional morphology of the adhesive glands of turbellarians because of their small size. Based on ultrastructural studies of worms fixed in the process of attachment to a substrate, the configuration of the two gland cell types in some species, and other evidence (see Tyler, 1988, for a clear summary), he ascribed functions to each cell type he identified and coined the term 'duo-gland adhesive organ'. This distinguishes these adhesive systems from other adhesion mechanisms such as specialized cilia or single gland cell types (Tyler, 1976, 1988).

In Tyler's duo-gland system, one kind of gland cell secretes dense, membrane-bound granules between 0.2 and 0.7 μm in diameter and is known as the *viscid gland* (Figures 5, 6, 8A) by Tyler (1976) based on evidence, albeit

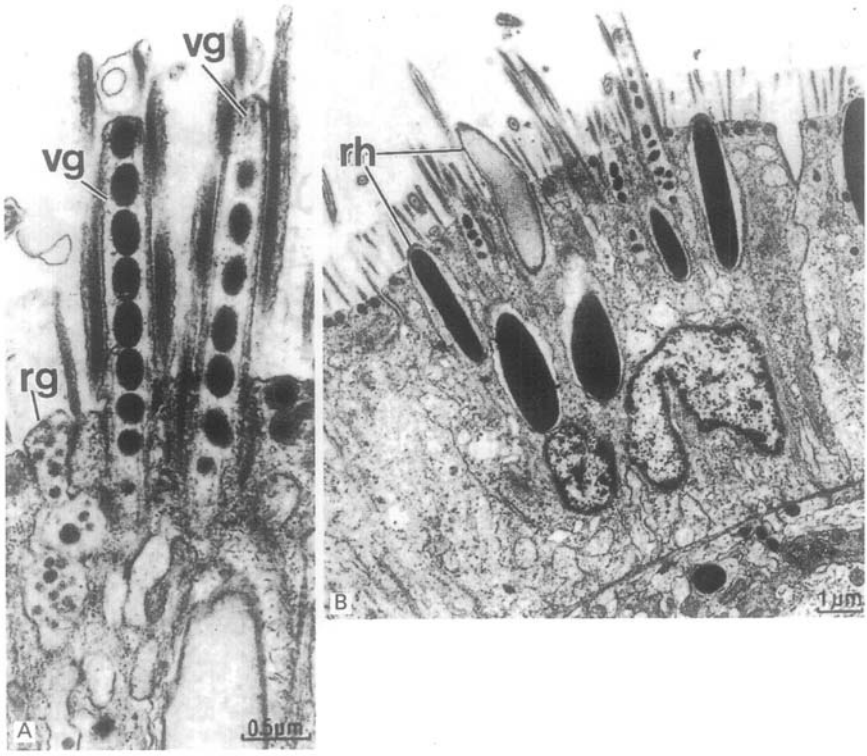


Figure 8 Transmission electron micrograph of the turbellarian *Messoplana falcata* (Neoophora: Rhabdocoela). A. Two viscid gland papillae (vg) and one releasing gland papilla (rg) in longitudinal section. Scale bar = 0.5 µm. B. A single anchor cell of an adhesive field contains rhabdites (rh) as well as viscid and releasing gland secretions. Scale bar = 1 µm. (Reproduced with permission from Tyler, S. (1976). Comparative ultrastructure of adhesive systems in the Turbellaria. *Zoomorphologie* **84**, 1–76. Springer-Verlag, Berlin, Heidelberg.)

circumstantial, that its secretion forms the adhesive. Tyler (1976) asserted that the smaller, less dense granules (diameter, approximately 0.1 µm) secreted by the second gland cell type disengages the adhesion generated by the products of the viscid gland, although this is also speculation, and was termed the *releasing gland* (Figures 5, 6, 8A). The third, non-glandular cell type is a modified epidermal cell with a well-developed cell web (Figure 5) and was termed the *anchor cell* by Tyler (1976) because its role is supportive (see also Tyler, 1988). The gland cell necks of the two kinds of secretory gland cells open through the anchor cell where microvilli with fibrous cores surround the gland cell necks like collars (Figures 5, 6). There may be small variations in the

local arrangement of gland cell types within species (Figures 6, 8). For example, most adhesive organs in *Haplopharynx* (Haplopharyngida) have two viscid glands and one releasing gland per anchor cell (see also Figure 8A for *Messoplana falcata* (Rhabdocoela)), but some organs were found to have three viscid glands and two releasing glands per anchor cell (Tyler, 1976). For further discussion concerning functional aspects of the duo-gland adhesive system, see Section 9.

According to Tyler (1976), five features common to all turbellarian duo-gland adhesive systems are: two gland cell types whose necks open onto the surface of the epidermis (Figures 5, 6, 8); one gland cell type secretes dense, membrane-bound granules and the other gland cell type secretes smaller, less dense, membrane-bound granules (Figures 6, 8); the gland cell necks penetrate one or more modified epidermal cells, or a region of syncytial epidermis, with a well-developed cell web and microvilli with fibrous cores that surround one or both gland cell necks (Figure 5); the unit comprising gland cells and epidermal component performs an adhesive role. A further nine additional characters, mostly concerning the anchor cell, were identified by Tyler (1976) as of potential phylogenetic and systematic importance because of their variability between taxa and these include: the number of gland cells of each type associated with each anchor cell (e.g. Figure 8A); whether gland cell necks have separate or common openings on the anchor cell surface; whether the gland cell necks branch; the ultrastructure and arrangement of the fibrous-cored microvilli.

In a further overview of the duo-gland adhesive system, Rieger *et al.* (1991) commented that in the Macrostomorpha, a common collar of specially strengthened microvilli surrounds the necks of the viscid and the releasing glands together whereas higher turbellarians have separate openings in the anchor cell for each gland 'neck' and microvilli line only the necks of the viscid glands.

For more detail of the structure and ultrastructure of the adhesive organs of the turbellarians that led to the proposal of Tyler's duo-gland hypothesis, Tyler (1976, 1988, and references therein) should be consulted. Studies have continued to chart the ultrastructure of the adhesive organs of a variety of free-living turbellarians. Examples of the duo-gland system referred to above include adhesive organs from a variety of sites on the bodies of the worms. The adhesive organs of: *Haplopharynx* (Haplopharyngida) are situated close to the main lateral nerves; *Myozona* (Macrostomida) form a distinct row along the lateral margins of the body but especially along the margin of the posterior tip; *Bradynectes, Microstomum* and *Paromalostomum* (Macrostomida) are along the length of the body; *Psammomacrostomum* (Macrostomida) and *Messoplana* (Rhabdocoela) are concentrated posteriorly; *Theama* (Polycladida) are arranged in a relatively large adhesive field on the ventral epidermis at the posterior end of the body; *Cicerina* (Rhabdocoela) are arranged

in four, evenly-spaced girdles along the length of the body plus a concentration of organs on a small, posterior tail plate; Otoplanidae (Proseriata) have a variety of organs posteriorly, ventrally and sometimes anteriorly; Tricladida form a glandular band on the ventral body margins (information extracted from Tyler, 1976). Ehlers and Sopott-Ehlers (1993) identified a duo-gland system for the caudal adhesive plate of *Jensenia angulata*, a member of the Dalyellidae in the Rhabdocoela 'Dalyellioida'. Therefore, unlike the Monogenea that tend to have their adhesive secretions deployed mostly anteriorly and/or posteriorly (Section 5), many turbellarians can release adhesives from several different sites.

4.5. Other Adhesive Systems in Free-living Turbellarians

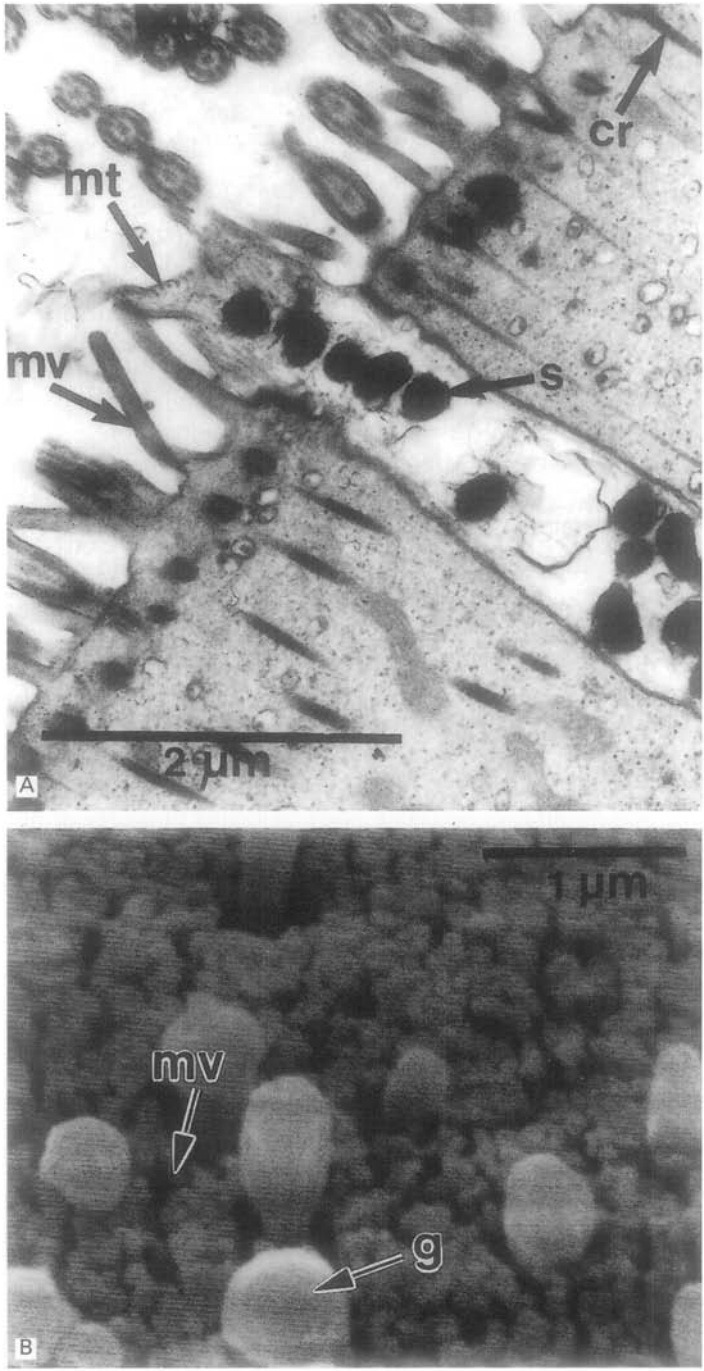
Despite the apparent widespread presence of the duo-gland adhesive system among turbellarian orders, Tyler (1976), Ehlers (1989), Rieger *et al.* (1991) and Ehlers and Sopott-Ehlers (1993) have drawn attention to the fact that this is not the only adhesive system in these flatworms. Rieger *et al.* (1991) noted that when adhesive glands were present in acoelomorph turbellarians, they appeared more like mucoid glands. Furthermore, some acoels can adhere to surfaces using specially modified cilia called haptocilia (Rieger *et al.*, 1991). Some larger triclads have a system assumed to be adhesive, but its relation to the duo-gland system is unknown: ovoid acidophilic granules with dense striated or reticular substructure are produced in marginal glands and in musculo-adhesive organs (Rieger *et al.*, 1991).

Ehlers' (1989) description of the adhesive system on the conical tail of the kalyptorhynch, *Schizochilus caecus*, demonstrated a major departure from the typical duo-gland system identified above. Two types of adhesive glands were distinguished based on the ultrastructure of their secretory bodies: type 1 gland cells produced larger, ovoid, denser granules than type 2 cells. Each gland cell possessed a neck, and a neck of gland cell type 1 always accompanied a neck of gland cell type 2. A row of longitudinal microtubules lined the necks as is commonly the case among turbellarians (Ehlers, 1989). Ehlers (1989) questioned whether this system conformed to the duo-gland system because both gland cell types had their own openings, he believed that both gland cell types were viscid glands and noted that an anchor cell was lacking. He speculated that perhaps the two secretions combined to form a special adhesive, but if this is the case how is detachment from a substrate mediated? This conundrum will reappear when the adhesive system of some adult monopisthocotylean monogeneans is reviewed in Section 5.2.2(e). Another departure from the duo-gland system in turbellarians is reported below among some rhabdocoels from the digestive tract of starfish (Jondelius, 1992, and Section 4.6).

The collectively termed frontal glands in some free-living turbellarians are of particular relevance to this review because of our interest in the anterior adhesive secretions of the Monogenea (e.g. Section 5.2). Unfortunately, according to Rieger *et al.* (1991) and Martínez-Alós *et al.* (1994), their function in turbellarians is unknown. Accumulations of these frontal glands can include mucous, rhabdite and rhammite glands and often the gland cells themselves can be elongate and/or conspicuous. The gland necks can be scattered at the anterior end of the worm, but may be grouped at discrete areas (Rieger *et al.*, 1991). The distinction is made by Rieger *et al.* (1991) that although sense receptors may be present near frontal gland necks, they are not specifically associated to form a glandulo-sensory complex. However it must be borne in mind that echinoderms have ciliated neurosecretory-like cells which are thought to release the de-adhesive component in their adhesive system (Flammang, 1996; Flammang *et al.*, 1994, 1998; Section 9).

The similarly named but differently arranged frontal organ of some acoelomorphs, according to Rieger *et al.* (1991), comprises a special set of mucoid frontal glands which open via a single pore between epidermal cells at the exact apical pole of the worm. Smith and Tyler (1985) described the simplest form for *Diopisthoporus gymnopharyngeus* (Acoela) which has two identical gland cells, the secretory granules of which appear lightly stained using standard transmission electron microscopy (TEM) procedures. The frontal gland necks have an electron-dense collar and longitudinally arranged microtubules. In some cases, however, the frontal organs in the Acoelomorpha are more complex. The term was redefined by Smith and Tyler (1986) as a collection of two to several large mucous-secreting glands (up to 38 gland necks were counted in *Otocelis* sp.) whose necks emerge together through a frontal pore at the exact apical pole of the body. Smith and Tyler (1986) also noted further elaborations in some species, with up to five additional types of gland cells, including rhabdoid and mucous glands, opening anteriorly but separate from the frontal pore.

Frontal glands were also detailed for *Nemertoderma* (Nemertodermatida) and *Paratomella* (Acoela) by Ehlers (1992): a variety of gland cells were described (four different types in *Nemertoderma* and three to four types in *Paratomella*, including rhabdoids) and the necks of each type were found near the anterior tip of the specimen but separated from each other. Smith and Tyler (1986) and Ehlers (1992) should be consulted for discussion and differing viewpoints of frontal organs and frontal glands. In the frontal glands of *Bothromesostoma personatum* (Typhloplanoida), Martínez-Alós *et al.* (1994) described two types of rhabdoid gland cells with long necks that extend anteriorly where the secretory bodies are released. It is unfortunate that the functional significance of frontal glands in turbellarians is unknown, but according to Rieger *et al.* (1991), they are assumed to be involved in attachment, defence or generation of slime for locomotion.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The homology or otherwise between the structure and ultrastructure of various adhesive system characters of turbellarians has been a topic of much research and debate, and this is reviewed in Section 4.9.

4.6. Adhesion in Symbiotic Turbellarians

The duo-gland adhesive system described above applies to several taxa of free-living turbellarians, including the Rhabdocoela 'Kalyptorhynchia' and Rhabdocoela 'Typhloplanoida' from Tyler (1976, 1988). Different systems appear to occur in symbiotic turbellarians. Studies by Jondelius (1992) on four species of the Pterastericolidae, a family of rhabdocoels symbiotic inside the digestive system of starfish, described the ultrastructure of a prominent complex of eosinophilic glands (Figure 9A) anterolateral to the pharynx that are thought to have an adhesive function. The gland cell necks ('processes' in the terminology of Jondelius, 1992) enter and open through otherwise normal epidermal cells, branching of ducts was not observed and microtubules line the periphery of the ducts (Jondelius, 1992, and Figure 9). Distally, the duct endings join the epidermal cells by septate desmosomes, and microvilli present in the region of duct endings are normal in the Pterastericolidae and do not have electron-dense cores characteristic of the duo-gland system (Figure 9A, and Jondelius, 1992). Secretory bodies in the four species of Pterastericolidae studied varied in electron density. There were slight variations in shape (elongate in *P. pellucida* and *Pterastericola* sp.; rounded in *P. fedotovi*; ovoid in *P. bergensis*) and size, but most were between 0.2 and 0.3 μm at their smallest up to 0.9 μm at their largest (Jondelius, 1992). Jondelius (1992) concluded that the glands were adhesive and most likely attached the worms to host epithelium. He considered that the secretory bodies in the Pterastericolidae were similar to the viscid gland granules of the duo-gland adhesive system, but that 'releasing glands' and their secretion were absent. It was suggested that the entosymbiotic lifestyle of these worms may dictate that they have no need for rapid release and repositioning like many of the free-living turbellarians that may be exposed, for instance, to predators. The pterastericolids, therefore, may have lost features of the duo-gland system (Jondelius, 1992). In view of this line of argument, it is also interesting to note that duo-glands are absent from species studied from the entosymbiotic rhabdocoel families Umagillidae, Graffillidae and Fecampiidae (see Jondelius, 1992).

Figure 9 Gland cell processes of the pterastericolid turbellarians, *Pterastericola* spp. (Rhabdocoela). A. Transmission electron micrograph of *Pterastericola fedotovi* showing secretory granules (s), microvilli (mv), microtubules (mt) and cilium rootlet (cr). Scale bar = 2 μm. B. Scanning electron micrograph of *P. pellucida* showing gland cell processes (g) and microvilli (mv). Scale bar = 1 μm. (Reproduced with permission from Jondelius, U. (1992). Adhesive glands in the Pterastericolidae (Plathelminthes, Rhabdocoela). *Zoomorphology* **111**, 229–238. Springer-Verlag, Berlin, Heidelberg.)

Frontal glands, already referred to above (Section 4.5), may be responsible for the attachment of two species of graffillids to their respective hosts. Kent and Olson (1986) reported a species of turbellarian ascribed provisionally to *Paravortex* from the skin and gills of *Zebrasoma flavescens*, a marine teleost fish, and noted frontal glands in the worm, although no mention was made of how attachment was achieved other than by protrusion of the pharynx. Similarly, Schell (1986) reported conspicuous frontal glands in *Graffilla pugetensis* from the pericardial cavity of a clam, *Macoma nasuta*. Histochemistry demonstrated the presence of mucosubstances and acid mucopolysaccharides in the frontal glands and Schell (1986) suggested two possible roles: provision of a mucoid coat for protection or sticky secretions for attachment.

Micropharynx parasitica is a marine planarian parasitic on the skin of a ray, *Raja radiata*, and uses a posterior attachment zone that attaches the worm 'deep in the host tissue, and often the animal cannot be removed without damaging it' (Ball and Khan, 1976). The implication of this observation, perhaps, is that some sort of pathological reaction may be stimulated by the secretion of an adhesive or histolytic substance, but no clear details of how *M. parasitica* achieves anchorage to its elasmobranch host are available.

Temnocephalans, most of which are ectosymbionts of freshwater decapod and isopod crustaceans, have some characteristic structures that are involved in attachment to their hosts: they possess a posterior disc for semi-permanent attachment and prehensile tentacles anteriorly used for attachment during locomotion and also for feeding (Figure 10). Sewell and Whittington (1995) provided an account of the low-looping locomotion of *Craspedella* sp. (now *C. pedum*; Cannon and Sewell, 1995) from the branchial chamber of the redclaw crayfish, *Cherax quadricarinatus*. Attachment of the posterior disc is achieved by a combination of suction and adhesion. Sewell and Whittington (1995) identified a granular secretion produced by posterior gland cells that leave a conspicuous, annular footprint on glass surfaces which reflects the position of the gland duct openings on the surface of the attachment disc. The gland cell bodies that produce the posterior adhesive secretion in *C. pedum* lie dorsally in the body, anterior to the caudal peduncle, and produce a finely granular secretion (Sewell and Whittington, 1995).

In a more detailed study of these secretions, Sewell (1997), using thick resin sections and TEM, identified four types of granular secretion associated with the posterior attachment organ of *C. pedum* (Figure 11) and none originated from rhabdites. Two types of electron dense granules were identified: the most abundant were large oval, elongate granules 0.9 μm long and 4.5 μm wide (sg1 in Figure 11A); less electron dense, circular to oval granules approximately 0.7 μm in diameter were less numerous (sg2 in Figure 11B) (Sewell, 1997). Two types of heteromorphic granules were also discovered: small (~0.2 μm in diameter) circular to oval granules of low electron density

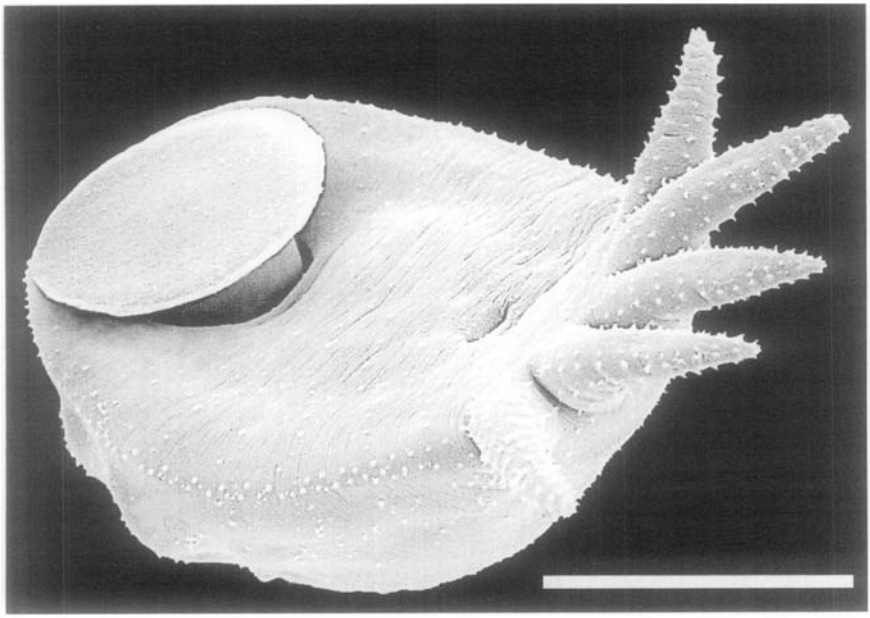
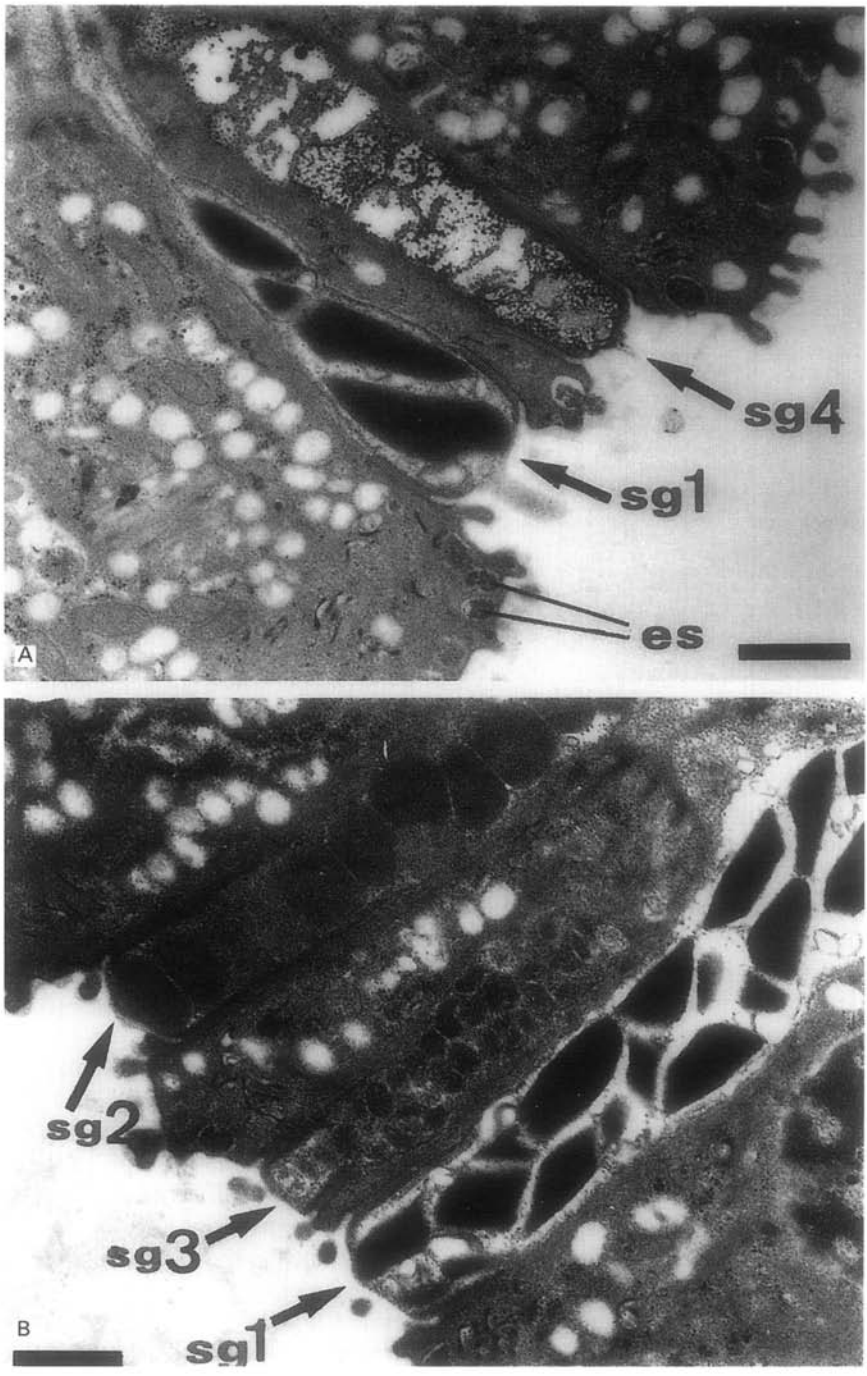


Figure 10 Scanning electron micrograph of *Craspedella pedum* (Temnocephalida) in ventral view. Scale bar = 200 μm. (Dr K.B. Sewell, original photograph reproduced with permission.)

(sg3 in Figure 11B) and large, mostly electron lucent irregularly shaped bodies (~0.6 μm across) containing small (~0.02 μm across) sparse, fine, electron dense granules (sg4 in Figure 11A) (Sewell, 1997). Other features of importance noted by Sewell (1997) include the observations that all four secretory types exited the ventral surface of the disc via separate pores and that no anchor cells were present (Figure 11). Clearly, based on this evidence, the posterior sucker of *C. pedum* does not possess a duo-gland adhesive system *sensu* Tyler (1976, 1988).

It has become entrenched in the literature, largely as a result of work by Williams on *Temnocephala novaezealandiae* (e.g. Williams, 1975, 1980, 1994), that all temnocephalans have a duo-gland system (Ehlers, 1985; Williams and Ingerfeld, 1988; Ehlers and Sopott-Ehlers, 1993). The notion of a duo-gland system for *T. novaezealandiae* was later modified when Williams (1994) changed her interpretation so that the entire posterior attachment disc was regarded as the duo-gland organ (i.e. 'developed to the macro-organ level of construction') without anchor cells. Thus, her interpretation was that the epidermal surface where the gland ducts open is fringed by modified microvilli (Williams, 1994). There has been a gradual realization, however, that


sg4
sg1
es
A
sg2
sg3
sg1
B

temnocephalans may present an arrangement that differs from Tyler's duo-gland system. Joffe *et al.* (1995a, b) suggested, albeit tentatively, that *Diceratocephala boschmai* and *Didymorchis* spp. have a 'modified duo-gland adhesive system' and that for *Didymorchis* spp. their posterior secretions are not 'wholly typical of the duo-gland adhesive system'. These data using TEM on the range of posterior secretions present in some temnocephalans uphold the suggestion of Sewell and Whittington (1995) that more ultrastructural studies are required among temnocephalans to understand their posterior adhesive system.

A study by Rohde and Watson (1995) reported gland ducts containing only two types of secretion in the posterior attachment organ of a *Temnocephala* sp., but it is possible that ducts containing other secretions were overlooked. It also seems likely that not all glands and their secretions at the posterior end of temnocephalans have an adhesive function. Posterolateral glands in *T. minor*, that open on the posterolateral margin of the worm to discharge rhabdoid secretory bodies (but not rhabdites; see Cannon and Watson, 1996), seem unlikely to be for adhesion. Cannon and Watson (1996) suggested that the secretion may be distasteful to predators or may perhaps act as a social pheromone.

At the anterior end where *C. pedum* bears five tentacles (Figure 10), Sewell and Whittington (1995) identified a sticky secretion exuded from them. Parenchymal rhabdite glands (rhabditogen cells of Jennings *et al.*, 1992) were located laterally in the body at the level of the gut and rhabdite tracts ran through the centre of each tentacle, but were most conspicuous in the central three tentacles (Sewell and Whittington, 1995). More pores were identified in the ventral than in the dorsal epidermis of all tentacles, and pores were most numerous in the distal concavities of the central three tentacles. The sticky secretion was classed as of rhabdite origin, although Sewell and Whittington (1995) cautioned that they did not observe intact rhabdites in the secretion of live worms. Since then, Sewell and Cannon (1995) have reported undischarged rhabdites protruding through the epidermis of a tentacle concavity of *Craspedella* sp. (now *C. pedum*; Cannon and Sewell, 1995). The rhabdites of *C. pedum* were considered to be true rhabdites (see Section 4.3) by Sewell and Whittington (1995), and Sewell (1997) further characterized them using TEM to be of the lamellate type (e.g. Figures 7A, B, C) of Smith *et al.* (1982). Furthermore, the rhabdites were the most prevalent secretions in the tentacles and were clearly concentrated about the distal concavity of the tentacles

Figure 11 Transmission electron micrographs of secretions from *Craspedella pedum* (Temnocephalida) opening onto the ventral surface of the posterior attachment disc. A. Gland ducts containing secretory products type 1 (sg1) and type 4 (sg4) and epitheliosomes (es). B. Gland ducts containing secretory products type 1 (sg1), type 2 (sg2) and type 3 (sg3). In both photographs, note the absence of specialized anchor cells *sensu* Tyler (1976). Scale bars = 0.5 μm. (Dr K.B. Sewell, original photographs reproduced with permission.)

(Sewell, 1997). Lamellated rhabdites have been recorded from several temnocephalan species: in *Didymorchis* sp. by Rohde (1987a), Rohde and Watson (1990) and Joffe *et al.* (1995a); in *Diceratocephala boschmai* by Joffe *et al.* (1995b); in *Temnocephala novaezealandiae* by Williams and Ingerfeld (1988); in *Notodactylus handschini* by Jennings *et al.* (1992).

Even last century, a role for rhabdites in the adhesion of temnocephalans was suggested (Haswell, 1888, 1893) but this notion was rejected by Williams (1980, 1992). The observations by Sewell and Whittington (1995) and by Sewell (1997), however, support Haswell's early assertion. Two other secretory types from the tentacles were identified by Sewell (1997) using TEM: a putative mucous secretion which was not concentrated in the area of the concavities, and a secretion associated with epitheliosomes of the tentacle epidermis. Williams (1975) considered that the epitheliosomes (termed 'secretion globules') had no adhesive role in *Temnocephala novaezealandae*, but suggested a role to coat, strengthen or change the permeability of the surface film on the worms (Williams, 1980). However, Sewell and Whittington (1995) considered that the epitheliosome-related secretion may have a role in releasing attachment by the tentacles.

Sewell (1997) noted that not all temnocephalans move by 'looping' but *Didymorchis* sp. and *Diceratocephala boschmai* move solely by ciliary gliding. This is an important observation because looping and therefore, by inference, the use of adhesive secretions, was a feature that has previously distinguished temnocephalans from other groups of turbellarians.

4.7. Adhesive Secretions on Turbellarian Eggs

There are cases in which some turbellarians and monogeneans (Section 5.5) use substances that appear to have adhesive properties to attach their eggs (taken here to mean a shelled zygote; after Kearn, 1986). Among turbellarians this seems to be a strategy employed by some temnocephalans. *Notodactylus handschini*, an ectosymbiont of the crayfish *Cherax quadricarinatus*, is an inactive worm and may spend many days (perhaps its whole life?) attached in the same location at the edges of the host's carapace. Adults are known to surround themselves with circles of their own eggs (Jennings *et al.*, 1992). These authors observed that this tactic protects the developing embryos because the adult worms seized any likely predators that settled near the eggs. The behaviour was considered a form of brooding by the parent. Jones and Lester (1992) mention briefly that eggs of another temnocephalan, *Diceratocephala boschmai* from the same crayfish host, also deposit their eggs on the decapod's carapace. Similarly, Sewell (1997) reported that *Craspedella pedum*, again from the same crayfish species, attaches its oval eggs individually and in a single layer by adhesive to

specific areas in the branchial chamber. Sewell (1997) noted that the base of the egg and the attachment cement remain attached to their substrate after the juvenile has hatched. To our knowledge, there has been no chemical analysis of the adhesives used by these, or any other, temnocephalans to attach their eggs to their host's surfaces.

4.8. Chemistry of Turbellarian Secretions

Our review has examined several secretory types from turbellarians that are implicated in adhesion but some, such as rhabdites, may have different functions depending on the taxon. Unlike the adhesives secreted by some of the larger invertebrates such as mussels, barnacles and echinoderms (see Sections 2, 3 and 9), the minute amounts of adhesive produced by the tiny gland cells of small turbellarians have precluded a thorough biochemical or molecular study. Histochemical tests, however, have been applied to some taxa and a valuable summary of this work was presented by Tyler (1988). The granular secretions of the viscid glands from three species of turbellarians, *Macrostomum hystricinum, Paromalostomum* sp. (Macrostomida) and *Polystyliphora* cf. *filum* (Neoophora: Proseriata), have proteinaceous and glycan moieties, with the glycans staining as polysaccharide or oligosaccharide and occurring perhaps as a glycoprotein. Further characterization by Tyler (1988) indicated that the viscid granules were rich in a basic protein (tests indicated this was due to arginine or histidine and not lysine), they reacted positively but equivocally for polyphenols (see Tyler, 1988, for details), were relatively rich in sulphydryl groups and were resistant to digestion by common proteases. Ultrastructural studies by Tyler (1988) using stains for vicinal diols (polysaccharides) indicated that the granules in the viscid gland secretion had a minor glycan component. Tyler (1988) noted that no test he applied demonstrated a reaction for secretions from the releasing glands.

Histochemical analysis of the rhabdites and other rhabdoids (see terminology of Smith *et al.*, 1982) of several turbellarian taxa was summarized in a comparative study of rhabdites by Smith *et al.* (1982). They noted that histochemical tests applied to the rhabdoids of acoels (see also Smith and Tyler, 1986) demonstrated their mucopolysaccharide nature, whereas rhabdites of representatives from Macrostomida, Polycladida, Proseriata and Tricladida (see Table II of Smith *et al.*, 1982) showed a negative reaction for some species to the carbohydrate-sensitive stains periodic acid-Schiff (PAS) and alcian blue, and were characterized by strong acidophilia. Jennings (1957) also found that rhabdoid bodies in turbellarians appear to be proteinaceous, giving negative reactions to PAS and alcian blue. Members of the Rhabdocoela also stain for acidophilic rhabdites. *Bothromestoma personatum*, a typhloplanoid representative of the Rhabdocoela, shows acidophilic staining for type I and type II

rhabdites found in the general body tegument, whereas the rhabdoid granules (true rhammites) are basophilic unlike other described rhammites (see Martínez-Alós *et al.*, 1994, for further discussion). Sewell (1997) characterized the rhabdites from the tentacles of *Craspedella pedum* (Temnocephalida) histochemically as a protein, lacking carbohydrate and other mucosubstances. Williams (1990) found acidophilic refractile rodlets which appear as typical rhabdites using light microscopy in female *Kronborgia isopodicola* (Rhabdocoela: Fecampiidae). The finely granular, posterior adhesive secretion in *C. pedum* responsible for securing the attachment disc to the host is strongly acidophilic and stains red with eosin, acid fuchsin and azocarmine (Sewell and Whittington, 1995).

4.9. Phylogenetic Considerations

The Platyhelminthes are regarded by many to occupy a pivotal position in the Animal Kingdom. Willmer (1990) commented that the flatworms may have provided the base from which higher metazoans of many different kinds launched. Since the Platyhelminthes comprise three major wholly parasitic assemblages and one largely free-living group, it is hardly surprising that the extant free-living turbellarians are such a focus for phylogenetic study. A good overview of turbellarian phylogeny is provided by Rieger *et al.* (1991). Since then, studies of platyhelminth phylogeny have continued and the most recent contributions are based largely on molecular evidence (e.g. Littlewood *et al.*, 1999; Litvaitis and Rohde, 1999). We restrict ourselves here to phylogenetic considerations of the Turbellaria based on their adhesive systems or related components such as rhabdites.

Almost all information distilled above about adhesives in turbellarians has contributed to the phylogenetic assessment of the group. The electron microscope was perceived as a major tool to provide more characters at the cellular and organelle level to supplement traditional morphological characters at the level of the whole organism. None of these studies, however, has produced a definitive phylogeny (Smith *et al.*, 1986). A useful summary of the studies in the fundamental paper by Tyler (1976), in which he proposed the duo-gland system for several taxa in the Turbellaria, is presented by Ehlers (1985) and incorporates some later publications.

The duo-gland system is present in the Macrostomida (+ Haplopharyngida), Polycladida, Proseriata, Tricladida and 'Typhloplanoida' (+ Kalyptorhynchia), but not in the Nemertodermatida, Acoela and Lecithoepitheliata (see Ehlers, 1985). Tyler (1988) later extended the systematic distribution of duo-glands by claiming their presence in the Lecithoepitheliata and in a freshwater species of *Microdalyellia*, a dalyellioid rhabdocoel turbellarian. Ehlers and Sopott-Ehlers (1993) described many duo-gland adhesive organs on the

caudal adhesive plate of *Jensenia angulata*, a free-living marine species of the Dalyellidae, this family becoming the only taxon within the Rhabdocoela 'Dalyellioida' to possess this characteristic. Essentially, therefore, Ehlers' opinion is that the duo-gland system (and also lamellated rhabdites) is an autapomorphy of the Rhabditophora (Ehlers, 1985, 1986; Jondelius, 1992).

Ehlers and Sopott-Ehlers (1993) claimed that a duo-gland system in *Jensenia* together with the presence of multilamellate rhabdites in many temnocephalids confirmed the concept that the monophylum Dalyellidae (Dalyellidae *sensu* Luther + Temnocephalida) possesses two characteristics inherited from stem species of the Rhabditophora. These characters were a typical duo-gland system including anchor cells (although anchor cells were considered no longer present in temnocephalids with their syncytial epidermis and muscular attachment organs) and lamellate rhabdites produced in subepidermal glands (Ehlers and Sopott-Ehlers, 1993). These two features (duo-gland system and lamellate rhabdites) were considered by Ehlers and Sopott-Ehlers (1993) to exist no longer in any other 'dalyellioid' taxon such as Provorticidae, Umagillidae, Graffillidae, Pterastericolidae and Fecampiidae, nor in the Neodermata (Section 10). We have noted above, however, that work by Sewell and Whittington (1995), Joffe *et al.* (1995a, b) and Sewell (1997) demands a careful re-examination of so-called duo-glands in the temnocephalids. Slight differences have been observed in the structure of the so-called duo-gland system among some turbellarians and these have caused Tyler to propose a closer relationship between the Polycladida, Proseriata, Tricladida and 'Typhloplanoida' (+ Kalyptorhynchia) (see Ehlers, 1985). Investigations by Jondelius (1992) on the Pterastericolidae, entosymbionts of starfish, led him to conclude that glands thought to have an adhesive function in these worms are not part of the duo-gland system and this fits with the general scheme discussed above.

In any analysis of phylogeny, a determination of homology versus analogy must be achieved. Homology can be defined as the similarity of structures in space and time due to common ancestry (see Rohde, 1990, for a summary), whereas analogy is similarity by virtue of convergence, especially through pressure to perform similar function (Tyler, 1988). In addition to structure, Tyler (1988) discussed the role of function in the assessment of homology and convergence, and used invertebrate adhesive organs as his example. Tyler (1988) concluded that structural and functional similarities among the duo-gland systems in many turbellarians (he highlighted the example of macrostomids and proseriates) suggested that their homology was highly probable, whereas structural differences, despite functional similarities, in the adhesive systems in other invertebrates was indicative of convergence. A brief comparison of the chemistry of the adhesive secretions used across macroinvertebrates is presented in Section 9 and demonstrates that there is no universal duo-gland mechanism common to the adhesive systems in marine organisms.

The problem of homology has been addressed with regard to frontal organs (a special arrangement of frontal glands characteristic of the acoelomorphs according to Rieger *et al.*, 1991) and/or frontal glands. The conclusion is that they are not homologous across taxa (e.g. between Acoela and Macrostomida; see Klauser *et al.*, 1986; Smith and Tyler, 1986) but are at least analogous and probably function to produce mucus for locomotion (Klauser and Tyler, 1987). Ehlers (1992), however, takes a different view and considers that like the Acoelomorpha, nearly all taxa of the free-living Rhabditophora have frontal gland complexes. Most glands of these complexes in all taxa of the Macrostomida are rhammite and rhabdite glands, unknown in the Acoelomorpha, and Ehlers (1992) proposed that these, especially the glands producing the true lamellate rhabdites of Smith *et al.* (1982), were 'evolutionary novelties' for the Rhabditophora and had been passed on to the Macrostomida and other rhabditophoran taxa.

Phylogenetic implications derived from rhabdites were addressed by Smith *et al.* (1982) and, at that time, there were few indications that these characteristic secretory bodies were concerned with adhesion. Three morphological variants of rhabdites were identified (Section 4.3) and Smith *et al.* (1982) concluded that there was a 'reasonably high probability' that these three types were homologous, but that the so-called rhabdites of acoels were not homologous with true rhabdites. Smith *et al.* (1982) emphasized that they were unaware of any rod-shaped secretory body that conformed to their definition of a rhabdite (Section 4.3) which occurred in any phylum other than the Platyhelminthes. Their detailed study of rhabdites indicated rhabdite homology in the Macrostomida, Polycladida, Proseriata, Tricladida, Rhabdocoela and Temnocephalida and thus agrees well with Tyler's (1976) previous assessment based on the duo-gland system. Since Smith *et al.* (1982), the apparent diversity of function among rhabdites within turbellarians has been demonstrated and some of these uses may include: repelling predators, capturing prey, forming cocoons, excreting metabolic wastes, producing mucus for ciliary gliding (Rieger *et al.*, 1991), protection by formation of scales (Jennings *et al.*, 1992) and adhesion (e.g. Sewell and Whittington, 1995; Sewell, 1997).

Taking all available evidence into account, Rieger *et al.* (1991) considered that Turbellaria were best described as comprising three clades: the Acoelomorpha, the Catenulida and the Rhabditophora, each a monophyletic group. For a different view, see Ehlers (1985, 1986, 1992). For current opinion of the phylogeny of the Turbellaria based on molecular evidence or molecular data meshed with all other available data (the 'total evidence' approach), see Litvaitis and Rohde (1999) and Littlewood *et al.* (1999) respectively.

4.10. Summary

As the foregoing sections have shown, there has been a significant volume of research on the adhesive systems among the Turbellaria. Studies by Tyler (1976, 1988) have contributed greatly to the development of the field of bioadhesion, especially his proposal of the duo-gland adhesive system that may also be present, in some form, in members of the Gastrotricha, Nematoda, Polychaeta and Echinodermata (see Flammang, 1996, and Section 9). Almost all studies on turbellarian adhesives, however, have had a phylogenetic basis. Functional investigations to examine the duo-gland system or other adhesive mechanisms among turbellarians are technically difficult because of their small size and Tyler's proposals for duo-gland adhesion (two antagonistic secretions: one an adhesive, the other a de-adhesive) remain speculation based on structure and ultrastructure. Indeed further characterization of adhesion in turbellarians, even using biomechanical, biochemical and/or molecular techniques, is likely to be precluded because the worms themselves and their adhesive glands are so tiny (Flammang *et al.*, 1998). Despite the fascinating characteristics of temporary adhesion displayed by many turbellarians (i.e. instant attachment and detachment; strong bonding between organism and substrate in a moist or wet environment), their small size entails that larger animals (macroinvertebrates) such as echinoderms provide more suitable and convenient candidates for study. Echinoderms, therefore, appear to be the 'system' of choice for present and future research to redress our poor understanding of the mechanisms of temporary adhesion (Flammang *et al.*, 1998). Further studies of functional and chemical aspects of temporary adhesion in relatively small invertebrates seem likely to move away from turbellarians, but may extend to, and focus on, their parasitic relatives, the Monogenea. The surfaces to which most turbellarians adhere are generally inert, whereas monogeneans adhere to their hosts' epithelial surfaces, a characteristic that would appear to place extra demands on their adhesive secretions.

5. ATTACHMENT BY ADHESIVES IN THE MONOGENEA

Monogeneans are generally strictly host-specific ectoparasites with a direct life cycle and live predominantly on the skin and gills of fish. Despite recent debate about whether or not the Monogenea are monophyletic (e.g. Justine, 1998, but see also recent phylogenetic hypotheses based on molecular analyses by Littlewood *et al.*, 1999, and Litvaitis and Rohde, 1999), the class is still considered to comprise two subclasses. These are the Monopisthocotylea (the subclass 'Polyonchoinea' in the terminology of Boeger and Kritsky, 1993,

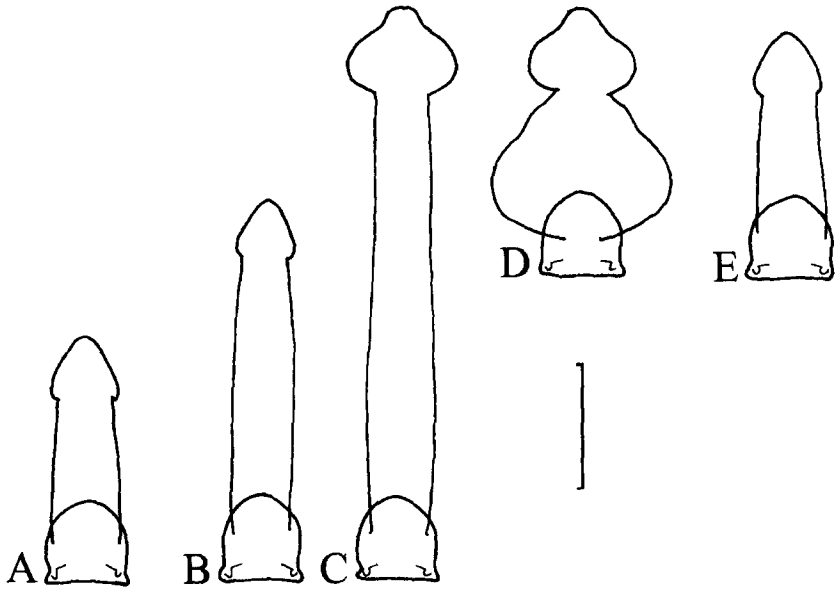


Figure 12 Diagram to demonstrate the looping locomotion of monogenean parasites based on observations of *Neoheterocotyle rhinobatidis* (Monopisthocotylea: Monocotylidae) moving across glass surfaces and epithelial surfaces of the gill lamellae of its elasmobranch host. A. The resting posture of the parasite with the posterior haptor bearing hooks attached firmly but the anterior end is detached and free to move around. B and C. Remaining attached firmly by the haptor, the parasite stretches its body forwards and can extend at least three times its resting length. The anterior end of the worm then attaches using anterior adhesive secretions. Note how the anterior end of the parasite flattens and spreads out as it attaches. D. Now attached firmly by the anterior end, the parasite releases attachment by the haptor, contracts the longitudinal muscles of the body and pulls the haptor forwards where it will reattach close to the head. E. After the locomotory step described, the monogenean severs the firm anterior attachment using adhesives and resumes its resting posture attached only by the haptor. Rapid forward progression is made by successive locomotory steps as described above. Scale bar = 750 μm. (I.D. Whittington and B.W. Cribb, original drawing.)

1997, in press) and the Polyopisthocotylea (which comprises two subclasses, the 'Oligonchoinea' and the 'Polystomatoinea' after Boeger and Kritsky, 1993, 1997, but Boeger and Kritsky (in press) propose a new subclass 'Heteronchoinea' to embrace the infra-subclasses 'Polystomatoinea' and 'Oligonchoinea'). Monopisthocotylea and Polyopisthocotylea are more widely accepted groupings and therefore we use them here. The monopisthocotyleans are a diverse group of flatworms that may parasitize most external skin surfaces, scales if present, fins, gills and nasal tissue of teleosts and chondrichthyes (Whittington, 1998). Some monopisthocotyleans live in

openings to, and ducts within, the urogenital tract, on surfaces of the viscera and inside the body cavity of elasmobranchs (Chisholm and Whittington, 1998a). The polyopisthocotyleans are a more homogeneous group that inhabit principally the gills of fishes, but one family, the Polystomatidae, has radiated in tetrapods from aquatic or semi-aquatic habitats and the adult worms

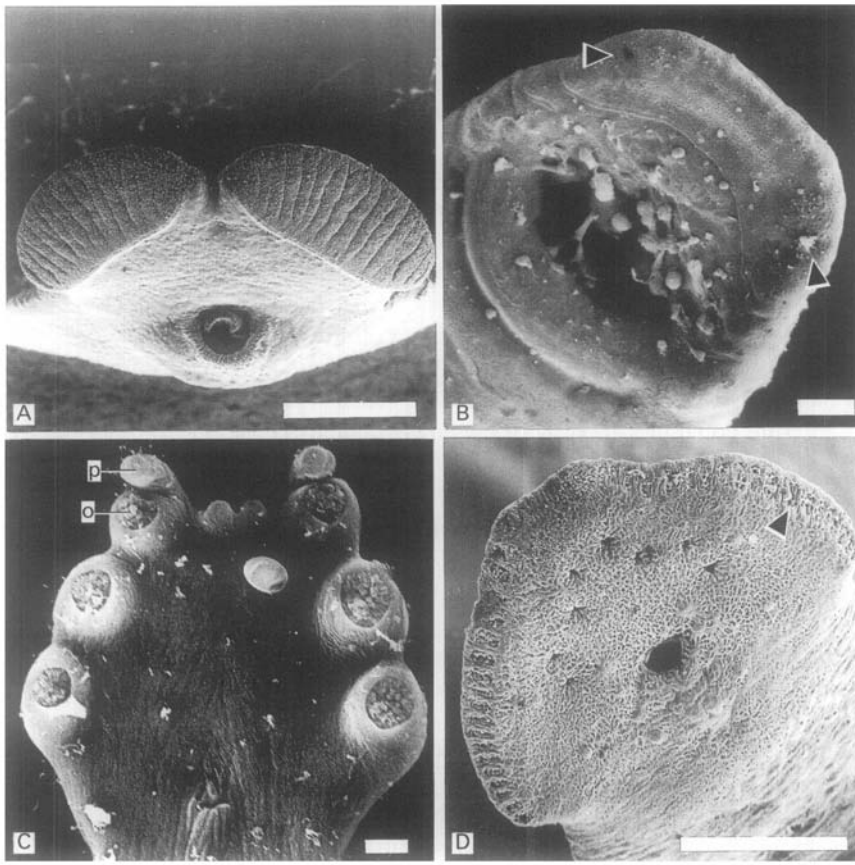


Figure 13 Scanning electron micrographs of the anterior ends of four monopisthocotylean monogenean parasites. A. *Entobdella* sp. (Capsalidae) has a pair of anterior ribbed 'diadems' through which the adhesive secretions emerge. Scale bar = 100 μm. B. The ventral surface of *Decacotyle lymmae* (Monocotylidae) has two small openings (arrowheads) through which adhesive is secreted. Scale bar = 10 μm. C. *Merizocotyle icopae* (Monocotylidae) has three pairs of large, ventral openings (o) through which adhesive is secreted. A pair of horn-like papillae (sensory?), one papilla (p) on each side of the head, is located in front of the most anterior adhesive opening. Scale bar = 10 μm. D. *Troglocephalus rhinobatidis* (Monocotylidae) has multiple adhesive outlets (arrowhead) at the ventral margins of the anterior end. Scale bar = 100 μm. (B.W. Cribb and I.D. Whittington, original photographs.)

parasitize internal sites connected to the exterior via openings (e.g. the urinary bladder, eyes, oral cavity) of amphibians and chelonian reptiles (Whittington, 1998).

Monogeneans, like the endoparasitic digeneans (Section 7), are well known for their leech-like looping locomotion (Figure 12) and it is hardly surprising that early zoologists grouped some flatworms with leeches (Kearn, 1998). Unlike leeches and digeneans, however, where movement is achieved mostly using a pair of strongly muscular suckers, monogeneans accomplish their locomotion by attaching alternately (Figure 12) their principal posterior attachment organ, the haptor (Figure 2), and their specialized anterior region (Figure 13). In the past, the term 'prohaptor' has been used to distinguish the anterior end from the posterior end, formerly known as the 'opisthaptor'. Now that the abbreviated term haptor has gained universal acceptance for the posterior end, we recommend that use of prohaptor be discontinued and be replaced by 'anterior end' or simply 'head'. We prefer and propose the term 'anterior adhesive areas' for the anterior regions of monogeneans specialized for attachment by adhesive secretions. As we highlighted in the Introduction (Section 1), several studies have investigated the functional morphology of the haptor for a variety of monogenean species, but only relatively recently has some attention focused on the functional morphology of the anterior adhesive areas.

5.1. Use of Adhesives by Monogeneans

Kearn (1998) noted the common perception that muscle-based locomotion by flatworms was slow, but stressed the speed and agility of the leech-like locomotion (Figure 12) of monogenean parasites. Indeed anyone who has faced the task of removing live monogeneans from fish skin surfaces or gill tissue will know of the frustration suffered as these nimble flatworms race away using their characteristic looping motion from needles, pipettes and other implements used to try and dislodge them. Kearn (1998) discussed the muscular processes involved in looping. Even though the anterior regions of relatively few monogenean species have been studied in detail, conspicuous anterior gland cells, especially in the Monopisthocotylea, are now known to secrete an adhesive or adhesives. Ultrastructural investigations of 15 species have been completed (Table 1) but a glance through taxonomic descriptions of most monopisthocotylean taxa demonstrates the presence of arrays of anterior gland cells (Section 5.2.2). Unlike accounts of new species of most adult cestodes and digeneans, therefore, the regular description of anterior gland cells in adult Monopisthocotylea indicates how conspicuous these structures are. Most monopisthocotyleans can move freely and rapidly over the host and while the haptor provides semi-permanent attachment, the anterior adhesive secretions provide firm but temporary attachment. The adhesives manufactured by the

anterior gland cells of monogeneans exhibit the following remarkable properties:

- worms stick *instantly* and firmly to the slimy, wet epithelial surfaces of their fish and other aquatic or semi-aquatic hosts despite continual and forceful water currents and even when the haptor is detached briefly during a locomotory step (Figure 12D);
- the tenacious adhesive bond is *reversible instantly* and anterior attachment is severed rapidly by parasites when the haptor resumes operation after a locomotory step (Figure 12E).

Secure attachment to a host is fundamental for the survival of any ectoparasite but is supremely important on fish because parasites may be dislodged when fishes swim. With a single known exception (Kearn and Whittington, 1992), adult monogeneans cannot swim and so their separation from host tissue is likely to result in parasite death. Shear forces on fish skin are considerable. Swimming motions of fishes demand movements of skin and fin surfaces, providing a hazard for any organism attempting to attach securely. The velocity of fish hosts provides further hazards to ectoparasites. Migrating sockeye salmon can maintain speeds of up to 3.2 km h^{-1} (Beamish, 1978), tuna can swim up to 42 km h^{-1} (Bone and Marshall, 1982) and rapid bursts of swimming activity up to 130 km h^{-1} (= 3610 cm s^{-1}) have been estimated for black marlin (Block *et al.*, 1992). Gill parasites are exposed continually to strong currents that ventilate the respiratory surfaces and some fast-swimming pelagic fish achieve irrigation of their gills by ram ventilation (Roberts, 1978; Bushnell and Jones, 1994), which must cause a considerable strain on any method of attachment. In the face of these apparent constraints, monopisthocotyleans in particular use their adhesives to prevent dislodgement when they change position (Figure 12) on their host for a variety of essential purposes. These include: larval migrations after initial invasion; finding new sites for feeding on epidermis; and seeking partners for cross-insemination (Kearn and Evans-Gowing, 1998).

In contrast, the majority of the gill-parasitic polyopisthocotyleans are highly mobile generally only as larvae or juveniles (Section 5.4.1) and may remain relatively sedentary when adult (Kearn, 1994; Whittington *et al.*, 2000b; Section 5.4.2). It is interesting that most taxonomic descriptions of adult polyopisthocotyleans rarely mention anterior gland cells that may function as adhesive glands, although it is possible that glandular tissue and associated ducts may be present but inconspicuous. Characteristically, the anterior end of adult Polyopisthocotylea comprises a terminal or sub-terminal mouth leading to an oral cavity (Kearn, 1994). The oral cavity can be enclosed by musculature and resembles an oral sucker, although there is no discrete and enveloping capsule surrounding the musculature as observed in suckers of, for example,

Table 1 A summary of the ultrastructure of the anterior secretions of monopisthocotylean monogeneans.

Family *Species*	S1 (rod-like bodies)[1]	S2 (spherical vesicles; type 1)[1]	S3 (spherical vesicles; type 2)[1]	'Non-adhesive' anterior secretion[1]	Reference
Acanthocotylidae					
Acanthocotyle lobianchi	180 ± 0.02 nm. Membrane-bound. Single layer of microtubules around forming S1 in cell. 6.7:1 (S1:S2 ducts)[2].	Electron-dense ≈125 nm. Not membrane-bound?[5]	None	None reported	Rees and Kearn, 1984
Capsalidae					
Benedenia lutjani	323 ± 11 nm. Membrane-bound. No microtubules surround secretion. 14 nm banding. 23:1 (S1:S2 ducts)[2].	Electron-dense 170 ± 10 nm. Membrane-bound.	None	Electron-dense 455 ± 29 nm roughly spherical secretion. Membrane-bound.	Whittington and Cribb, 1999
B. rohdei	306 ± 8 nm. Membrane-bound. No microtubules surround forming secretion. 14 nm banding. 9:1 (S1:S2 ducts)[2].	Electron-dense 153 ± 8 nm. Membrane-bound.	None	Electron-dense 574 ± 37 nm. roughly spherical secretion. Membrane-bound.	Whittington and Cribb, 1999
Entobdella soleae	≈300 nm. Membrane-bound. Microtubules around forming S1 in cell. No banding reported. 1.3:1 (S1:S2 ducts)[2].	Finely granular electron-lucent ≈550 nm.[3] Membrane-bound.	None	Electron-dense 300 nm[3] roughly spherical secretion. Membrane-bound.	El-Naggar and Kearn, 1983

Table 1 cont.

Family *Species*	S1 (rod-like bodies)[1]	S2 (spherical vesicles; type 1)[1]	S3 (spherical vesicles; type 2)[1]	'Non-adhesive' anterior secretion[1]	Reference
E. australis	213 ± 14 nm. Membrane-bound. Microtubules around forming S1. 12 nm banding. 2:1 (S1:S2 ducts)[2].	Electron-dense 117 ± 9 nm. Not membrane-bound.	None	None reported	Whittington and Cribb, 1998a
Entobdella sp.	213 ± 9 nm. Membrane-bound. Microtubules possibly around forming S1. 12 nm banding. 3.3:1 (S1:S2 ducts)[2].	Electron-dense 153 ± 12 nm. Not membrane-bound.	None	None reported	Whittington and Cribb, 1998a
Dactylogyridae					
Dactylogyrus amphibothrium	≈310 nm.[3] Membrane-bound. Single layer of microtubules around forming S1 in cell. No banding reported.	Electron-dense ≈520 nm.[3] Membrane-bound.	Electron-lucent vesicles ≈286 nm.[3] Membrane-bound.	None reported	El-Naggar and Kearn, 1980
D.hemiamphibothrium	≈325 nm.[3] Membrane-bound. Single layer of microtubules around forming S1 in cell. No banding reported.	Electron-dense. Membrane-bound.	Electron-lucent vesicles. Membrane-bound.	None reported	El-Naggar and Kearn, 1980
Bychowskyella pseudobagri	Electron-dense 'elliptical' body[4] – not rod-like body. Not membrane-bound?[5] No banding reported.	Electron-dense elliptical body with more-electron-dense core. Not membrane-bound?[5]	None	None reported	Yuan and Long, 1996

Table 1 cont.

Family *Species*	S1 (rod-like bodies)[1]	S2 (spherical vesicles; type 1)[1]	S3 (spherical vesicles; type 2)[1]	'Non-adhesive' anterior secretion[1]	Reference
D. aristichthys	Electron-dense 'elliptical' body[4] - not rod-like body, but only one secretion can exit from duct at a time. Membrane-bound. Homogeneous (no banding). S1smaller diameter than S2.	Medium electron-dense elliptical body. Not membrane-bound?[5]	Electron-lucent elliptical body. Not membrane-bound?[5]	None reported	Yuan and Long, 1996
Silurodiscoides sp.	Electron-dense 'elliptical' body[4] - not rod-like body, however only one secretion can exit from a duct at a time. Not membrane-bound?[5] No banding reported.	Electron-dense elliptical body. Not membrane-bound?[5]	None	None reported	Yuan and Long, 1996
Gyrodactylidae *Gyrodactylus eucaliae*	≈450 nm. Membrane-bound. Single layer of microtubules around forming S1. No banding reported.	Fine particulate electron-lucent body 500–600 nm. Discontinuous membrane.	Electron-dense flattened oval 1.5–9 μm (?) (figs. show 1.4 μm maximum diam.). Tripartite membrane.	None reported	Kritsky, 1978

Table 1 cont.

Family *Species*	S1 (rod-like bodies)[1]	S2 (spherical vesicles; type 1)[1]	S3 (spherical vesicles; type 2)[1]	'Non-adhesive' anterior secretion[1]	Reference
G. sprostonae	≈300 nm.[3] Membrane-bound. Microtubules surround forming S1. No banding reported.	Electron-dense oval secretion ≈325 nm.[3] Membrane-bound.	Electron-lucent spherical secretion ≈370 nm.[3] Membrane-bound.	None reported	Yuan and Lang, 1997
Monocotylidae *Monocotyle spiremae*	355 ± 60 nm. Not membrane-bound. No microtubules observed around forming S1. 12 nm banding.	None observed	None	Electron-dense ≈822 × 406 nm. Regular substructure observed. Some fragments of membrane observed.[3]	Cribb *et al.*, 1997
Merizocotyle australensis	309 ± 18 nm. Membrane-bound. No microtubules around forming S1. 11 nm and 143 nm banding. More S1 than S2 secretion.	Electron-dense 130 ± 6 nm. Not membrane-bound.	None	Electron-dense, elongate 374 ± 23 nm long. Not membrane-bound.	Cribb *et al.*, 1998

[1] All measurements are diameters unless stated otherwise.
[2] Ratio of ducts containing rod-like secretion (S1) to ducts containing spherical secretion (S2).
[3] Measured from micrograph(s).
[4] Micrographs show elliptical bodies at high magnification and in one plane. It is possible that these are rod-like bodies in transverse section. This dual morphology is apparent in the lower magnification photograph of figure 3, plate I in Yuan and Lang (1997).
[5] No report of being membrane-bound but not confirmed as not membrane-bound.

digeneans (see Agrawal *et al.*, 1996). Following Pearson (1992), this structure has been termed a 'false sucker' (Whittington *et al.*, 1989; Pichelin *et al.*, 1990; Agrawal *et al.*, 1996). According to Whittington *et al.* (1989), it is doubtful that any monogeneans possess a true oral sucker, although there is no doubt that their false suckers can generate considerable suction. As an alternative to a false sucker, the anterior end of other adult polyopisthocotyleans may be provided with a pair of internal buccal suckers as in the mazocraeideans (Kearn, 1994, 1998). The little that is known about adhesive secretions in adult polyopisthocotyleans is reviewed in Section 5.4.2.

The presence of gland cells at the posterior end of monogeneans is also known and, in some cases, hooks have been lost in adult parasites and attachment is achieved solely by adhesion (e.g. in *Leptocotyle minor*, see Kearn, 1965; in *Anoplodiscus australis*, see Roubal and Whittington, 1990; Section 5.3). There are also several reports in the literature of drops of viscous secretions on the surfaces of monogenean eggs or on their appendages and some of these are thought to have adhesive properties (Section 5.5).

Compared with turbellarians, there are considerably fewer studies on glands and their secretions in monogeneans. In addition to anterior adhesive glands (also referred to as cephalic, oral or buccal glands (see Fried and Haseeb, 1991) or 'head organs'), monogeneans may possess other glands variously described as digestive, pharyngeal or oesophageal. These open at different sites along the foregut and are considered to be associated with extracellular digestion (Fried and Haseeb, 1991). In later sections on cestodes (Section 6) and the Digenea (Section 7), we review briefly the structure and possible functions of numerous gland cell types at the anterior ends of various stages in the life cycle of these parasitic flatworms. For the Monogenea, we have concentrated almost exclusively on the relatively few detailed studies of gland cells that play a role in adhesion.

5.2. Adhesion at the Anterior End of Monopisthocotylean Monogeneans

5.2.1. *Larvae*

The larvae of the Monogenea, including their gland cells, have been reviewed extensively by Whittington *et al.* (2000b), to which the reader is referred for more detail. Anterior gland cells are conspicuous in most larvae (= oncomiracidia) at the level of the light microscope and we use the larva of the monocotylid *Monocotyle spiremae* (Figure 14), examined in detailed by Chisholm and Whittington (1996a), to demonstrate their arrangement and contents for a typical monopisthocotylean. The gland cells described in all larval monogeneans are uninucleate and each cell bears a single gland duct. Two

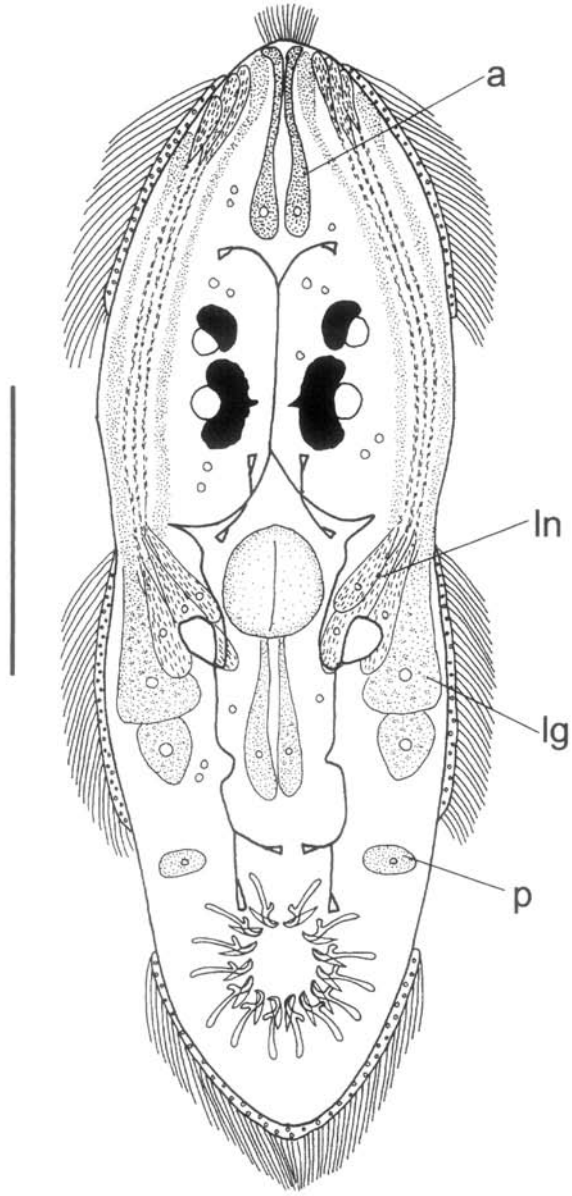


Figure 14 Oncomiracidium of *Monocotyle spiremae* (Monogenea: Monopisthocotylea: Monocotylidae) showing the following gland cell types as observed using phase contrast light microscopy: anteromedian gland cells (a), lateral gland cells containing granular secretion (lg), lateral gland cells containing needle-like secretion (ln) and posterior gland cells (p). Scale bar = 50 μm. (Modified from Chisholm and Whittington, 1996a.)

anteromedian gland cells containing dense granular secretion open at the anterior margin of the head (Figure 14). Two large gland cells containing less dense granular secretion are located on each side of the body at a level posterior to the pharynx. Their relatively wide gland ducts lead anteriorly and open at the lateral borders of the head on either side of gland duct openings, carrying needle-like secretion (Figure 14). A group of three gland cells containing needle-like secretion is located on either side of the pharynx and their ducts travel anteriorly and open on the lateral margins of the head between the gland duct openings containing granular secretion (Figure 14). In *M. spiremae*, there are two posterior gland cells, one on each side of the body near the haptor, that contain granular secretion (Figure 14), but ducts or pores associated with these posterior gland cells, and those in other monocotylid larvae, have not been observed (Chisholm and Whittington, 1996a). Possible homology between gland cells in a larval and adult monocotylid, *Heterocotyle capricornensis*, was demonstrated by Chisholm and Whittington (1996b). Studies of anterior gland cells of other larval monopisthocotyleans using light microscopy demonstrate differences in their number and arrangement, dependent on family, but two gland cell types producing granular or needle-like secretions, each with separate gland ducts, are a consistent feature with few exceptions (Whittington *et al.*, 2000b).

The ultrastructure of the anterior gland cells and their secretions have been studied in the oncomiracidia of only two species of monopisthocotyleans. Rod-shaped bodies are the most abundant secretory type in the larva of *Entobdella soleae* (Capsalidae; see El-Naggar and Kearn, 1983) and these equate to the needle-like secretions described using the light microscope for this species by Kearn (1974) and in other species by numerous investigators (Whittington *et al.*, 2000b). A second, less abundant and spherical secretory type was also described in larval *E. soleae* using TEM (El-Naggar and Kearn, 1983). All gland ducts that convey each secretory type open into three adjacent adhesive sacs on each side of the larva, an arrangement that may permit the two secretory types to mix (El-Naggar and Kearn, 1983). Ducts containing the spherical secretory bodies open with a single aperture, whereas those conveying rod-like bodies open with multiple apertures (El-Naggar and Kearn, 1983) and these features persist in adults (Section 5.2.2 and Figure 16B). Secretory bodies in the anterior region of the larva of the monocotylid, *Neoheterocotyle rhinobatidis*, are similar when studied by TEM. Rods are present but two different types of spherical secretions were identified: large granules are thought to equate with the contents of the anteromedian head glands, whereas smaller granules are thought to relate to the lateral granular gland cells (e.g. Figure 14) (Whittington *et al.*, 2000b). Since the ultrastructure of anterior glands in the larvae of only these two monopisthocotylean species has been studied, there is clearly a need for more investigation.

There can be little doubt that secretions from the anterior gland cells of most

larval monogeneans are used for attachment. Unlike anterior secretions in larval and juvenile cestodes and in miracidia and cercariae of digeneans, which, depending on their particular life cycle, may have a role in penetration of a host (Sections 6 and 7), the direct life cycle of monogeneans places emphasis on successful host location and then host invasion (Whittington, 1997) by the oncomiracidium. This requires an effective means of attaching to a host once located. It has been demonstrated that larvae of some monogeneans are infective immediately after hatching (Kearn, 1981; Whittington *et al.*, 2000a) and Whittington *et al.* (2000b) provide examples of the oncomiracidia of several species known to attach to, and detach from, glass surfaces by their anterior end in the same manner as adult parasites. However, not all anterior gland cells of larval monopisthocotyleans have been implicated as adhesives. Kearn (1970) suggested that the anteromedian gland cells containing granular secretion in larval monocotylids (e.g. Figure 14) may soften the opercular cement of the egg before hatching. The two ultrastructurally different types of spherical secretory bodies identified by Whittington *et al.* (2000b) in larval *N. rhinobatidis* (Monocotylidae) may lend this hypothesis some support. Similarly, disappearance of the anterior median gland cells in all stages of the capsalid, *Trochopus pini*, found on its host's gills indicates that these glands' role is completed soon after attachment (Kearn, 1971b).

The invasion route by larvae of two monopisthocotyleans, *Amphibella torpedinis* (Amphibdellatidae) and *Calicotyle kroyeri* (Monocotylidae), into their elasmobranch ray hosts is unknown. Adult *Amphibdella* grow and mate in the heart (Euzet and Combes, 1998) and juvenile *C. kroyeri* are reported from the rectal gland (Kearn, 1987a). An invasion route suggested by Kearn (1987a) for *C. kroyeri* larvae was penetration of host tissue, possibly through thin gill epithelium, and then transport via the blood system to the rectal gland. Although there is no evidence for this, Kearn (1987a) suggested the anterior median head glands could aid penetration. Euzet and Combes (1998) did not speculate about the invasion route by larval *Amphibdella* but a similar route to that proposed for *C. kroyeri* is possible. Further study may demonstrate that some anterior secretions in larvae of these two monogenean species may have a histolytic function.

5.2.2. *Adults*

(a) *Anterior gland cells identified by the light microscope.* An increasing number of taxonomic descriptions of adult monopisthocotyleans from various families contain information not only about the presence of anterior gland cells, but also details of their contents as distinguished at the level of the light microscope. Thus, some members of the following genera in a range of families are reported to possess anterior gland cells of two different types, one

producing needle-like or rod-shaped bodies and the other producing granular or spherical bodies: Dactylogyridae (*Dactylogyrus*, see Gerasev, 1977; *Cichlidogyrus*, see El-Naggar and Khidr, 1985; *Schilbetrema*, see El-Naggar, 1985; *Quadriacanthus*, see El-Naggar and Serag, 1986; *Protoancylodiscoides*, see El-Naggar, 1987; *Neocalceostomoides*, see Whittington and Kearn, 1995); Gyrodactylidae (*Macrogyrodactylus clarii*, see El-Naggar and Serag, 1987; *M. congolensis*, see Arafa, 1998); Monocotylidae (*Empruthotrema*, see Kearn, 1976; *Heterocotyle* and *Neoheterocotyle*, see Chisholm and Whittington, 1996b and 1997 respectively; *Decacotyle*, see Chisholm and Whittington, 1998b; *Merizocotyle*, see Chisholm and Whittington, 1999); Pseudodactylogyridae (*Pseudodactylogyrus*, see El-Naggar *et al.*, 1993).

In some monopisthocotyleans, the distinction between two different types of anterior gland cells is less clear. Kearn (1993) described the anterior adhesive secretion of *Enoplocotyle kidakoi* (Enoplocotylidae) to consist of conspicuous needle-shaped secretory bodies, but commented that the ducts of some live specimens also contained granular secretory bodies. During the development of some capsalids, the so-called posterior median head glands that contain needle-like bodies display a massive increase in size whereas growth of lateral gland cells, which also contain needle-like bodies, either ceases or continues slowly (e.g. *Trochopus pini*, see Kearn, 1971b; *Benedenia seriolae*, see Kearn *et al.*, 1992).

(b) *The diversity in morphology of the anterior adhesive areas.* There is considerable variation in the way the anterior gland ducts are deployed at the head region and in the morphology, structure and arrangement of the anterior adhesive areas. Figure 13 demonstrates a diversity of anterior morphologies across two monogenean families, Capsalidae and Monocotylidae, that parasitize skin, gill and nasal surfaces of elasmobranch rays. Variation among the anterior adhesive areas in dactylogyrids was reviewed by El-Naggar and Kearn (1980), who described two pronounced lobes on each anterolateral region of the head in *Dactylogyrus amphibothrium*. Two lobes are also known for *D. extensus* (see Gerasev, 1977) and *Schilbetrema aegyptica* (see El-Naggar, 1985), but three lobes are reported on each side of the head in *D. hemiamphibothrium* (see El-Naggar and Kearn, 1980). Three anterior lobes on each side of the head is the more common condition for dactylogyrids (see also *Cichlidogyrus*, see El-Naggar and Khidr, 1985; *Quadriacanthus*, see El-Naggar and Serag, 1986; *Protoancylodiscoides*, see El-Naggar, 1987; *Neocalceostomoides*, see Whittington and Kearn, 1995) and is also known for the Pseudodactylogyridae (see El-Naggar *et al.*, 1993). Whether or not the anterior adhesive areas of monopisthocotyleans are lobed, the presence of three apertures, three adhesive sacs or three specialized regions on each side of the head, into or onto which the contents of the anterior adhesive glands are secreted, is common. For example, dactylogyrids that possess only two head lobes on each side of the anterior end still have three eversible adhesive sacs on

each side of the head (El-Naggar and Kearn, 1980). Figure 13C demonstrates this 'tripartite' arrangement on each side of the head for *Merizocotyle icopae* (Monocotylidae) and three apertures, sacs or specialized regions are also known for capsalids (e.g. *Entobdella soleae*, see Kearn and Evans-Gowing, 1998). The tripartite arrangement also extends to acanthocotylids because, according to Rees and Kearn (1984), although there is only one adhesive sac on each side of the head in *Acanthocotyle lobianchi*, each sac contains three distinct, eversible adhesive lobes. Rees and Kearn (1984) proposed that the seemingly common arrangement of six separate points for anterior adhesion to host surfaces by monopisthocotyleans may provide firm attachment to resist shear forces exerted by water currents from different directions. It was argued that a tripartite arrangement on each side of the head may also resist rotation should adhesion be achieved by outlets on only one side of the anterior end.

A single pair of adhesive apertures, one on either side of the head, was noted above for acanthocotylids. This condition is also present in enoplocotylids, udonellids and gyrodactylids. Indeed similarity between acanthocotylids and enoplocotylids, including the presence of a single adhesive sac on either side of the anterior end, prompted Kearn (1993) to propose the Acanthocotyloidea to contain these two families. Udonellids are a group of flatworms whose position has long been debated, but *Udonella* is now considered to be a monogenean (Littlewood *et al.*, 1998, 1999). Adult udonellids possess one anterior adhesive sac on each side of the head and each contains a 'cushion' onto which opens a dense array of duct openings from gland cell bodies close to the pharynx (Kearn, 1998). These cushions can be everted from the adhesive sacs and, according to Nichols (1975), the resulting adhesion attaches the parasite to a substrate. This arrangement is reminiscent of the single aperture containing eversible lobes in acanthocotylids (Rees and Kearn, 1984) and the single pair of adhesive sacs, one on each side of the head, in gyrodactylids (Kritsky, 1978; Yuan and Lang, 1997). El-Naggar (1992, 1993) examined the anterior end ('head lobes' in his terminology) of *Gyrodactylus groschafti* and *Macrogyrodactylus clarii* respectively using scanning electron microscopy (SEM). He determined that each adhesive sac of *G. groschafti* contains eight to 12 papillae, while each sac of *M. clarii* contains at least 12 papillae. All papillae are microvillous and ducts of the anterior adhesive glands open onto the papillae (El-Naggar, 1992, 1993). The ultrastructure of the anterior regions of these gyrodactylids has not been examined. It seems likely, however, to resemble that described for the monocotylid, *Merizocotyle australensis*: numerous tubular projections are covered in microvilli (Cribb *et al.*, 1998). Ducts from two different gland cell types open onto the tubular projections inside each of three apertures on either side of the head (Cribb *et al.*, 1998).

Some monogeneans, however, possess many adhesive outlets on each side

of the head. Figure 13D shows that multiple adhesive apertures occur in members of the Monocotylidae (see also *Neoheterocotyle* described by Chisholm and Whittington, 1997 and *Monocotyle spiremae* studied by Cribb *et al.*, 1997). Species of *Troglocephalus* (Figure 13D) and some *Monocotyle* species are relatively large (greater than 6 mm long) and it seems likely that an increase in parasite size may require more adhesive outlets. However, specimens of *Neoheterocotyle* are relatively small monogeneans (approximately 2 mm long; Chisholm and Whittington, 1997) and therefore their size and large number of adhesive outlets do not fit this idea. An alternative hypothesis is that the number of anterior adhesive outlets may have evolutionary implications. Acanthocotylids are considered by some to be primitive monogeneans (e.g. Llewellyn, 1970, 1982; Rees and Kearn, 1984) and have a single opening on each side of the head. Udonellids have a similar arrangement, but the relative placement of this group within the Monopisthocotylea is presently unresolved (Littlewood *et al.*, 1999). Rees (1986) reported that the duct openings from anterior adhesive glands in the microbothriid, *Leptocotyle minor*, another group considered primitive, were arranged around the sub-terminal mouth. A more in-depth study to relate the morphology of the anterior end to the evolution of monogeneans is required (see also Section 5.8).

Rees and Kearn (1984) commented that all ancestral monogeneans may have had the ability to withdraw their anterior adhesive areas into a sac when not in use, as observed for acanthocotylids, dactylogyrids, gyrodactylids (after El-Naggar, 1992, 1993) and possibly in some monocotylids (Cribb *et al.*, 1998). The arrangement in groups like capsalids where the adhesive areas are exposed permanently (e.g. *E. soleae*, see Kearn and Evans-Gowing, 1998; *Benedenia* spp., see Whittington and Cribb, 1999) may be a derived condition (Rees and Kearn, 1984). Advantages to monogeneans that possess permanently exposed adhesive surfaces may include: enhanced speed of attachment; increase in surface area for attachment; and energetic savings avoiding the need for protruding and retracting eversible lobes (Rees and Kearn, 1984). Advantages to parasites that have the ability to retract their anterior adhesive areas or those that possess a cavity or reservoir behind each adhesive outlet (e.g. Cribb *et al.*, 1997) include: the ability for two or more different secretory types to mix before release to the substrate; provision of space to store adhesive immediately prior to secretion; and prevention of accidental adhesion to detritus such as sand particles and detached host tissue during a locomotory step.

(c) *Observations on the anterior end when moving across surfaces.* There are several accounts of how different monopisthocotyleans move across host surfaces or on substrates such as glass (Kearn, 1987b, 1988; Whittington and Barton, 1990; Cribb *et al.*, 1997, 1998; Whittington and Cribb, 1999). In general, the descriptions are similar and looping is depicted in Figure 12. A common feature is that the anterior adhesive areas spread thinly and flatten

considerably, especially in regions where the apertures or outlets of the anterior gland ducts are located (e.g. Cribb *et al.*, 1998). The inference is that the surface area of the anterior end of the parasite increases where the adhesive secretions are released and provides, therefore, a larger area of contact at the interface between worm and host. It was noted above that six apertures for release of adhesive secretions is a common arrangement. We have observed that specimens of *Benedenia* spp. (see Whittington and Cribb, 1999) can achieve firm attachment to glass and to host surfaces using adhesive secretions from only a single 'attachment zone' on their disc-like anterior attachment organs, even in the presence of considerable water currents. Attachment involving some, but not all, of the anterior apertures was also noted in *Monocotyle spiremae* (see Cribb *et al.*, 1997). We have made similar observations for other monogenean species from the Capsalidae, Dactylogyridae, Dionchidae, Gyrodactylidae and Monocotylidae (Whittington and Cribb, unpublished observations). It is studies such as these that demonstrate how tenacious the anterior adhesives secreted by monogeneans are.

(d) *Ultrastructure of the anterior gland cells and their secretory bodies.* The most appropriate and detailed way to study the anterior adhesive areas of monogeneans is by using electron microscopy (Figures 13, 15). Currently, the ultrastructure of the anterior adhesive secretions of 15 species of monopisthocotylean monogeneans has been studied from five families (Acanthocotylidae; Capsalidae; Dactylogyridae; Gyrodactylidae; Monocotylidae; Table 1). Glandular systems comprising one, two and three different types of gland cells and their secretory bodies, which are distinct ultrastructurally, have been identified and are summarized in Table 1. With the exception of a single study on three species of dactylogyrids by Yuan and Long (1996), the presence of rod-shaped bodies is consistent and their ultrastructure is similar for all species studied (Whittington and Cribb, 1999, and Table 1). Yuan and Long (1996) described elliptical bodies in a single species from each of the following dactylogyrid genera: *Bychowskyella, Dactylogyrus* and *Silurodiscoides* (Table 1). The absence of rods in these species represents a major difference from all other studies and we consider it likely that these elliptical secretions may indeed be rod-shaped bodies, but sectioned obliquely (Table 1; Figure 15C). Rod-shaped secretions often show a banded periodicity (Table 1; Figure 15B), but are not like lamellate rhabdites of turbellarians (Section 4.3 and Figure 7). It is unknown whether rod-shaped bodies of monogeneans have a discrete length or whether they form a continuous, thread-like secretory body because of difficulties with the plane of sections (Figure 15D).

Another type of anterior adhesive secretion identified in monopisthocotyleans comprises spherical bodies, but these may differ in electron density between species (Table 1). For example, spherical bodies are electron-lucent in *Entobdella soleae* (see Kearn and Evans-Gowing, 1998) and electron-dense in *Benedenia* spp. (see Whittington and Cribb, 1999). Spherical bodies identified

by TEM most likely equate with the granular secretions observed using light microscopy. As mentioned earlier, the more abundant needle-like bodies observed in light microscopy studies most probably relate directly to rod-like bodies observed using TEM. If this is the case, it demonstrates that rods are probably the most abundant secretory bodies at the anterior end of most monopisthocotyleans (see also ratios of ducts carrying different secretory body types in Table 1). For this reason, it has been assumed that rods provide the

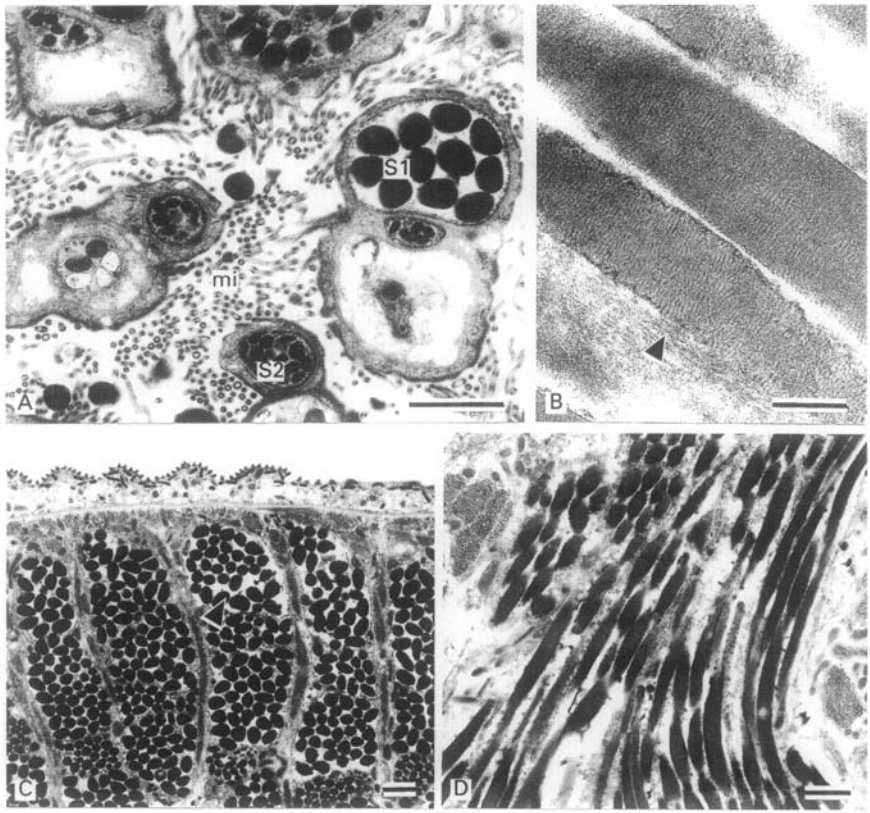
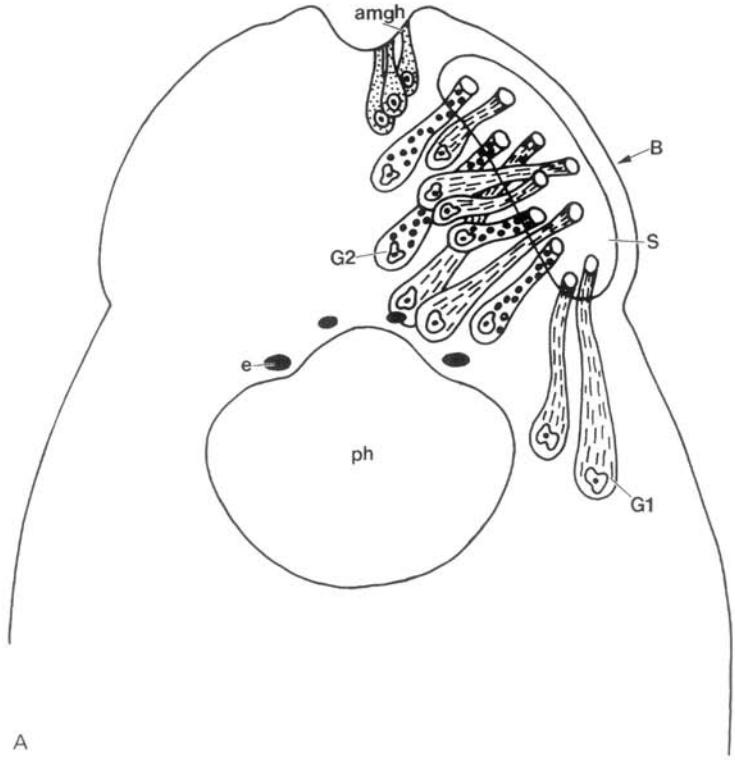


Figure 15 Transmission electron micrographs of secretory bodies found in the gland ducts which open through the adhesive area of *Merizocotyle australensis* (Monogenea: Monopisthocotylea: Monocotylidae). A. Two types of secretory bodies, rod-like (S1) and spherical (S2), are found in separate ducts but are adjacent to each other where the ducts open. Microvilli (mi) are visible. Scale bar = 1 μm. B. The rod-shaped secretion shows fine banding (arrowhead). Scale bar = 200 nm. C. In transverse section, the rod-shaped secretion looks spherical or elliptical and the bodies are densely packed into the ducts (arrowhead). Scale bar = 1 μm. D. Longitudinal sections through the rod-shaped secretion shows parallel stacking. Scale bar = 1 μm. (B.W. Cribb and I.D. Whittington, original photographs.)

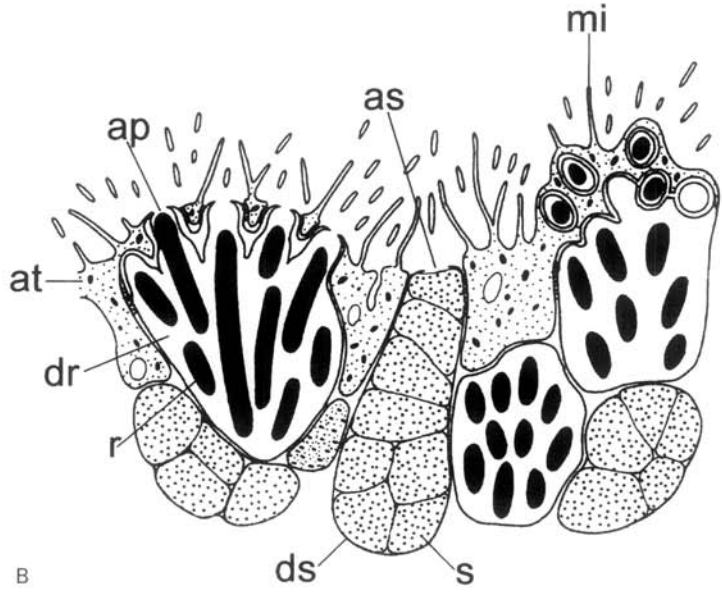
bulk of the adhesive material in monopisthocotyleans and the report of only rods at the anterior end of *Monocotyle spiremae* by Cribb *et al.* (1997) supports this hypothesis.

An important feature of rods is that, during their formation (e.g. El-Naggar and Kearn, 1983), each secretory body is enclosed by a single layer of microtubules that lie parallel to each other and to the long axis of the rod. This is reported in all cases except for the monocotylids and *Benedenia* spp. (Table 1). In those studies where microtubules around rods have not been noted, it is possible that sections were not cut through rods sufficiently early in their development (Cribb *et al.*, 1997). El-Naggar and Kearn (1980) proposed that encircling microtubules may be contractile and could play a role in transporting products from different parts of the cell to help in assembly of secretory bodies. Another suggestion was that microtubules may not only orientate rods within the gland cells to the lumen of their gland duct, but also may help maintain the parallel arrangement of rods into bundles (El-Naggar and Kearn, 1980). Microtubules have not been reported in the cytoplasm of cells producing spherical bodies nor around the forming bodies themselves (Table 1). Other differences noted between different secretory products identified and characterized across different monopisthocotylean species include whether or not rods and spherical secretory bodies are membrane-bound and whether the spherical secretion is electron-dense or electron-lucent (Table 1).

(e) *Hypotheses on possible interactions between different secretory types.* In monopisthocotylean monogeneans with two or more different types of anterior gland cells, the ducts of these cells mingle and will allow the secretions to mix (Figure 16). However interactions proposed between the different secretory types are speculations. It has been hypothesized that rods may provide the adhesive material, regulated by a second (El-Naggar and Kearn, 1983) and, sometimes, a third secretion (e.g. gyrodactylids; Table 1) responsible for rapid de-adhesion. Alternatively, a second secretion may interact with rods to produce the adhesive (Rees and Kearn, 1984). The discovery of only a single rod-shaped secretory type in *Monocotyle spiremae* by Cribb *et al.* (1997) supports our hypothesis that rods are the primary adhesive component in monogeneans and perhaps indicates that a second secretion is not necessary for adhesion (Whittington and Cribb, 1998a). However, different groups of monogeneans may possess different adhesive systems. Using TEM on *Merizocotyle australensis* (Monocotylidae), Cribb *et al.* (1998) discovered that the two secretion types were extruded simultaneously. Most of the adhesive matrix was formed from the contents of rods and the second secretion was observed at the edges and throughout the matrix of the extruded adhesive as an electron-dense material. This can be taken as evidence that rods and spheres in *M. australensis* react together in some way to generate the adhesive bond.



A



B

Kearn and Evans-Gowing (1998) used specimens of *Entobdella soleae* (Capsalidae) preserved at intervals during attachment and detachment to study the interplay between different secretory types. They determined that of two secretory types, the rods contributed the bulk of the cement, but the second, spherical secretory bodies changed in their duct ends immediately before attachment. It seems that the contents of the spherical bodies in *E. soleae* are released from duct endings as a liquid and spread rapidly to infiltrate and react with secreted rods. This sequence of events is remarkably rapid and precise. Knowledge of, and evidence for, how monopisthocotylean monogeneans detach from a substrate with rapidity and precision is scant. The possibility that a second secretion may act as an 'unsticking' agent was suggested above. Kearn and Evans-Gowing (1998) generated an alternative proposal that implicates a role for the specialized tegument that surrounds the anterior adhesive areas of monopisthocotyleans (Figures 16B, 17). They suggest that this special tegument is important in severing the adhesive bond by, in some way, dissolving the adhesive immediately adjacent to the attachment site. Tegument of the anterior adhesive areas differs from the general body tegument (Figures 16B, 17). It contains different inclusion bodies (Figure 17) and bears numerous short microvilli (Figures 15A, 16B, 17; see also Kritsky, 1978; El-Naggar and Kearn, 1980, 1983; Rees and Kearn, 1984; Yuan and Long, 1996; Yuan and Lang, 1997; Whittington and Cribb, 1999).

The presence on the specialized anterior adhesive area tegument of short microvilli that differ from longer microvilli reported from elsewhere on the body (e.g. Lyons, 1970), suggests that these structures have some importance in attachment by adhesion. El-Naggar and Kearn (1980) remarked that the short microvilli may provide a means of increasing the surface area for bonding between parasite and host. It is interesting that Tyler (1976) commented

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Figure 16 The anterior end of *Entobdella soleae* (Monogenea: Monopisthocotylea: Capsalidae) compiled from studies using light and transmission electron microscopy. A. Diagram showing the distribution and contents of the anterior median head glands (amgh), gland cells containing rod-shaped bodies (G1), gland cells containing spherical vesicles (G2) and the arrangement of the gland cell ducts. Note that the gland duct openings from the different gland cell types intermingle where they open through the specialized adhesive area tegument (S). For clarity, gland cells are shown on one side of the head only. The full complement and extent of gland cells are not depicted and gland cells are not drawn to scale. e, Eye; ph, pharynx. The arrow labelled B indicates the plane through which the section in (B) was taken. (Modified and redrawn from El-Naggar and Kearn, 1983). B. Diagram reconstructed from TEM sections through the surface of an adhesive pad of *E. soleae* (see arrow labelled B in Figure 16A). The aperture (ap) of the ducts carrying rod-shaped bodies (r) demonstrate the 'pepperpot-like' nature of the duct endings (dr) containing the rods. The aperture (as) of the ducts (ds) carrying spherical vesicles (s) is a single opening. Note the specialized adhesive area tegument (at) and associated microvilli (mi). (Modified from Kearn and Evans-Gowing, 1998.)

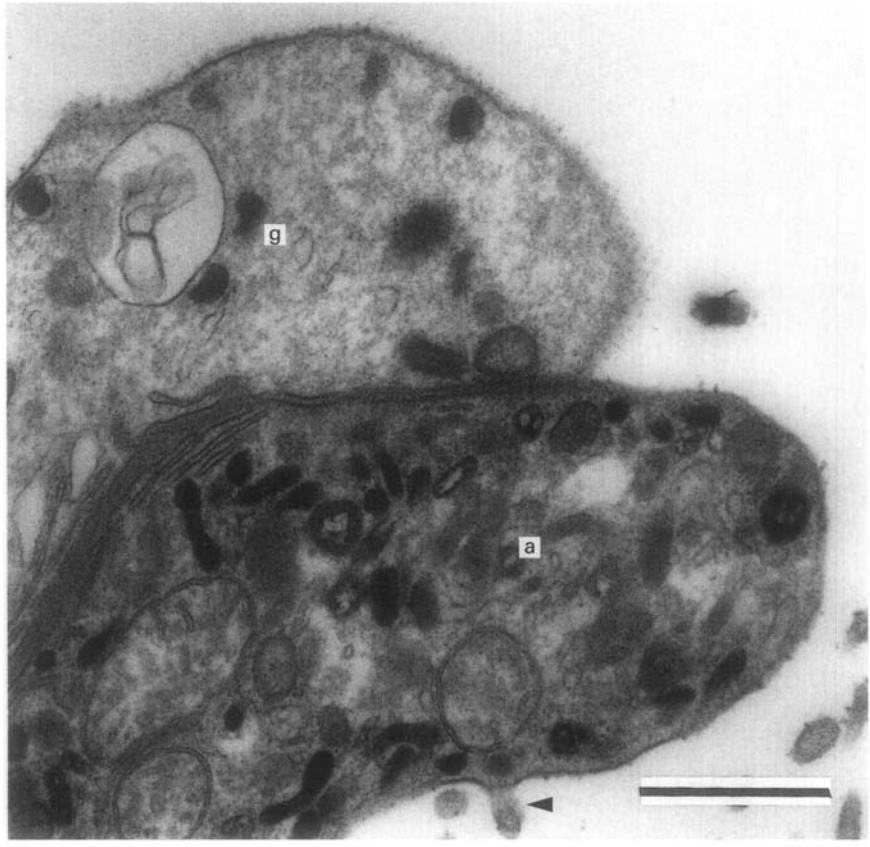

Figure 17 Transmission electron micrograph showing the general body tegument (g) and adjacent adhesive area tegument (a) of *Benedenia rohdei* (Monogenea: Monopisthocotylea: Capsalidae). Note the difference in the electron-density and the inclusion bodies between the different tegument types. The adhesive area tegument is microvillous (arrowhead). Scale bar = 500 nm. (B.W. Cribb and I.D. Whittington, original photograph.)

that microvilli on the anchor cells of turbellarians possessing a duo-gland adhesive system (Section 4.4) contact the substrate; he considered that the microvilli were the sites of adhesion in this group. An alternative or perhaps additional function proposed for the microvilli on the anterior adhesive areas of monogeneans was that they may mix the different secretory products together and perhaps spread the adhesive onto the host's surface (Lyons, 1970). There is, however, no evidence that the anterior microvilli are motile.

(f) *Non-adhesive anterior secretions?* All of the anterior glands discussed

above usually open into or onto specialized regions, mostly located anteroventrally, and where more than one secretory type is present, there is the possibility that some of these secretions may interact (Section 5.2.2(e)). Table 1 shows that other gland cells have also been identified at the anterior end of some monopisthocotyleans, but these cells do not open into the adhesive areas. For example, in *M. australensis*, a third secretion is released ventrally between the large apertures shown in Figure 13C (Cribb *et al.*, 1998). Similar additional anterior secretions, spatially distinct from those opening into the adhesive areas, have been described in *E. soleae* (the anterior median head glands; see Figure 16A) by El-Naggar and Kearn (1983), in *Monocotyle spiremae* (the anteromedian gland that opens dorsally) by Cribb *et al.* (1997) and in *Benedenia* spp. (at the anterodorsal extremities of the body proper) by Whittington and Cribb (1999). Their separate location indicates that these secretions are unlikely to be involved in adhesion. A possible function ascribed to these additional anterior secretions in *E. soleae* was release of pheromones for mate recognition (El-Naggar and Kearn, 1983), but Cribb *et al.* (1997) have stated that this possibility is far less likely for gill parasites such as *M. spiremae*. There is a need for further study of these anterior gland cells.

5.3. Adhesion at the Posterior End of Monopisthocotylean Monogeneans

5.3.1. *Larvae*

Posterior gland cells in larvae have been studied only at the level of the light microscope. It was mentioned above (Section 5.2.1) that larvae of *Monocotyle spiremae* (Monocotylidae) have two posterior gland cells containing granular secretion, one on each side of the body, near the haptor (Figure 14). Neither ducts nor pores associated with them in this or other monocotylid larvae have been observed (Chisholm and Whittington, 1996a). The larvae of other monopisthocotyleans such as the capsalids *E. soleae* and *E. hippoglossi*, possess four gland cells containing granular secretion that lie posteromedianly in the body with ducts that pass posteriorly into the haptor (Kearn, 1974). The oncomiracidium of the microbothriid, *Leptocotyle minor*, has especially well developed posterior gland cells (Kearn, 1965). With the exception of the study on *L. minor* by Kearn (1965), there is a lack of detailed information on the posterior glands of larvae whose adults are known to rely largely or entirely on haptoral attachment by adhesion. Larvae of *Udonella* (Udonellidae) apparently use adhesives secreted from gland cells in their haptor for attachment (Schell, 1972) and there is no sign of hooklets or other posterior sclerites at any stage in their development (Kearn, 1998).

5.3.2. *Adults*

The haptor of monogeneans is generally considered to be an organ that achieves mechanical attachment to host surfaces using, in the case of monopisthocotyleans, hooklets, hamuli and/or suction. There are reports, however, that some adult monopisthocotyleans either supplement mechanical attachment using adhesives or rely entirely on glandular secretions for adhesion by the posterior end. In some cases, adult monogeneans appear to have dispensed with haptoral armature entirely and adult udonellids and microbothriids fall into this category.

Udonellids attach to the hard carapace of their copepod hosts using adhesive secretions from the posterior end of the body. Ivanov (1952) described an abundance of posterior gland cells and he was certain that attachment was achieved by secretions rather than suction. It would seem that hooklets or other sclerites are useless for attachment to the carapace of an arthropod. Rohde and Watson (1995) described that the surface of the posterior attachment organ of *Udonella caligorum* was separate from the general body tegument and was covered by densely packed microvilli extending not from the tegument, but from the basal lamina. Numerous gland ducts opened between the microvilli and two secretory types were identified: large or small dense ovoid bodies (Rohde and Watson, 1995). We are unaware of any observations on live adults that recount the speed of attachment and detachment by the haptor of udonellids.

Another monogenean family for which haptoral hooks appear to be ineffective is the Microbothriidae which live attached to the hard, enamel-like placoid scales of sharks (Kearn, 1965). The small haptor of *L. minor* attaches to host denticles by an adhesive secretion (termed 'cement' by Kearn, 1965) and an adhesive residue remains on glass surfaces where attached parasites were located. Observations by Kearn (1965) showed that *L. minor* can move in a leech-like fashion using adhesive glands at the front and back of the animal, but detachment of the haptor took several seconds as it stripped away slowly from the host denticle. This may be consistent with detachment occurring simply by secretion of fresh adhesive to permit the slow withdrawal of the attached haptor from a substrate. An unpublished study by Rees (1986) suggested that the posterior adhesive secretion in *L. minor* is produced by a single type of gland cell. The slowness of the detachment process by the posterior end of *L. minor* prompted Rees (1986) to suggest a chemical difference between anterior and posterior adhesive systems in microbothriids and this deserves further study.

Posterior adhesion by another microbothriid, *Dermophthirius carcharini*, was described by Rand *et al.* (1986). They noted that adhesion, via an acellular tyrosine-rich lipoprotein, may be enhanced by a scale-like pattern on the surface of the adhesive secretion and by longitudinal furrows on the inside

ventral surface of the haptor, both of which complement the dorsal surface of the denticle. Rand *et al.* (1986) suggested that there may also be a role for suction. These authors noted that the posterior secretion in *D. carcharini* may travel along the length of the haptoral microvilli, but pointed out that this function of transporting secreted material had not been proposed for microvilli. If this observation by Rand *et al.* (1986) is substantiated by further study, it represents a different mechanism by which adhesive secretions in the Monogenea can be delivered to surfaces for attachment.

Like udonellids and microbothriids, adult anoplodiscid monogeneans also attach themselves to their hosts using haptoral adhesives but unlike udonellids and microbothriids, hooklets occur in larvae of *Anoplodicus* but are lost in

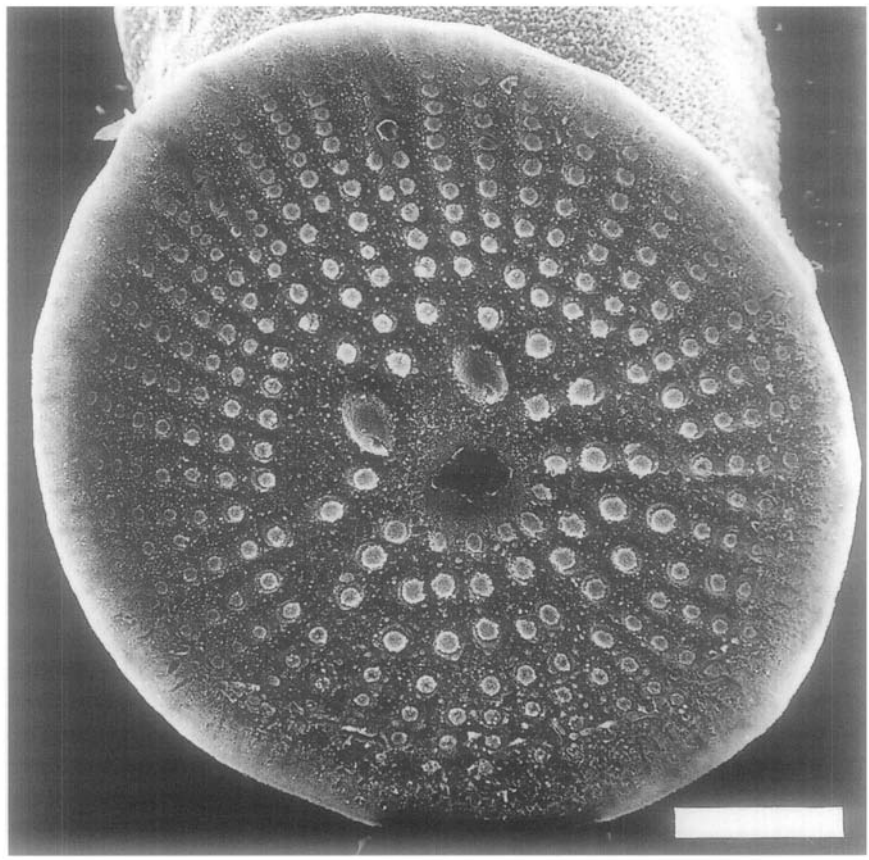

Figure 18 Scanning electron micrograph of *Trimusculotrema uarnaki* (Monogenea: Monopisthocotylea: Capsalidae) showing the haptor covered by papillae. Scale bar = 100 μm. (I.D. Whittington and B.W. Cribb, original photograph.)

adults (Roubal and Whittington, 1990). It appears that the posterior secretions of *A. australis* erode epidermis from the host fin rays and the monogenean then adheres directly to the fin ray (Roubal and Whittington, 1990).

The haptor of most capsalid monogeneans generates suction. Attachment is assisted by the large anterior hamuli that impale host epithelium; the peripheral hooklets fasten the edge of the haptor to host tissue and a thin fringing membrane, the marginal valve, prevents an influx of sea water (e.g. Kearn, 1964). For *Trimusculotrema*, however, Kearn (1994) has suggested that its haptoral morphology (Figure 18) indicates that it is incapable of generating suction and attached worms needed to be peeled from glass surfaces implicating use of adhesives. No ultrastructural study has been made of the haptor of this capsalid genus.

So-called 'dactylogyroideans', which usually possess four hamuli arranged in two pairs orientated in opposite directions (dorsal and ventral; see Figure 2B) that counter-rotate to impale adjacent secondary gill lamellae, have complex haptors (Kearn, 1994). Their mechanical attachment may also be supplemented by additional mechanical devices such as the squamodisks of diplectanids and anterior spines in some dactylogyrids (Kearn, 1994), but use of adhesive secretions is also known. Kearn and Whittington (1994) noted that the dactylogyrid, *Hamatopeduncularia pearsoni,* has reduced hamuli and produces an adhesive from posterior gland cells with ducts that lead into the haptor. It can attach to sites in the branchial chamber other than between gill lamellae and, unlike most other dactylogyroideans, can attach to glass surfaces using the haptor (Kearn and Whittington, 1994). There is evidence, however, that haptor glands may be relatively widespread in the Dactylogyridae and El-Naggar and Kearn (1989) and Kearn and Gowing (1989) reported two gland types, haptor surface glands and hamulus glands, in *Cichlidogyrus halli typicus* and *Tetraonchus monenteron* respectively. The haptor surface glands are unicellular, lie in the posterior region of the body, produce coarsely granular secretory bodies and their microtubule-lined ducts open at various sites on the posteroventral surface of the body proper and on the dorsal and ventral surfaces of the haptor. The hamulus glands are multinucleate, have cytoplasmic extensions that contact the proximal surfaces of the hamuli and their secretion is stored in reservoirs, one of which is associated with each hamulus point (e.g. Kearn and Gowing, 1989). The spherical secretion emerges from the haptor through the aperture from which the hamuli tips protrude and so the products of this gland bathe the distal ends of the large hamuli. The function(s) of these gland cells is unknown, but it has been suggested that the haptor surface gland secretion may be a temporary adhesive to stabilize the haptor after a locomotory step (e.g. Kearn, 1987b) and before hooklets and hamuli are established securely. El-Naggar and Kearn (1989) and Kearn and Gowing (1989) suggested a histolytic function for the hamulus glands to help the penetration of host gill epithelium by the hamulus tips.

Kearn (1998) has suggested that a separate development of adhesives may have occurred in the Dactylogyridae, highlighted by the discovery by Kearn *et al.* (1995) that *Neocalceostomoides brisbanensis* attaches to gill tissue using a thin, membraneous haptor. The adhesive appears to be produced by intra-haptoral cytons and is exocytosed onto the ventral haptor surface (Kearn *et al.*, 1995). Hamuli in this parasite are reduced to a single pair, directed ventrally, as are all the hooklets. The thin, foliaceous and adhesive haptor of *N. brisbanensis* is reminiscent of the membraneous but highly folded, posterior rosette organ of gyrocotylideans (Section 6.3) that is also known to secrete adhesive material.

5.4. Adhesion in Polyopisthocotylean Monogeneans

5.4.1. *Larvae*

Fewer comprehensive studies of gland cells in larval polyopisthocotyleans exist but Whittington *et al.* (2000b) review these. Figure 19 illustrates the arrangement and content of gland cells for the larva of *Grubea cochlear* (Mazocraeidae) to demonstrate the main features for a polyopisthocotylean as determined using light microscopy. A set of gland cells on either side of the pharynx and a second set laterally on either side of the body posterior to the pharynx, both with gland ducts leading anteriorly to open around the terminal mouth, all contain needle-like secretion in oncomiracidia of this family (Figure 19; Whittington and Kearn, 1990). No ultrastructural studies have been made to determine whether these needle-like secretions are electron-dense rod-shaped bodies like those reported from the anterior glands of most larval and adult monopisthocotyleans (Section 5.2). Most polyopisthocotylean larvae possess anterior gland cells that contain either needle-like secretion or granular secretion, but the latter is the most common condition reported so far (Whittington *et al.*, 2000b). Only a single species studied, *Tonkinopsis transfretanus* (Bychowskicotylidae), has been reported at the level of the light microscope to have both of these different types of secretions, but this requires confirmation (Whittington *et al.*, 2000b).

The ultrastructure of the anterior glandular secretions of a single polyopisthocotylean species, *Zeuxapta seriolae* (Axinidae), has been studied and this revealed that there are two different types of granular secretions based on granule size (Whittington *et al.*, 2000b). There are, however, no descriptions of the anterior gland cells of *Z. seriolae* using the light microscope and, until these are forthcoming, the ultrastructural studies on these anterior glands are of limited value (Whittington *et al.*, 2000b).

Posterior gland cells with granular contents were described in the larvae of two hexabothriid species by Whittington (1987). Peripheral ducts containing

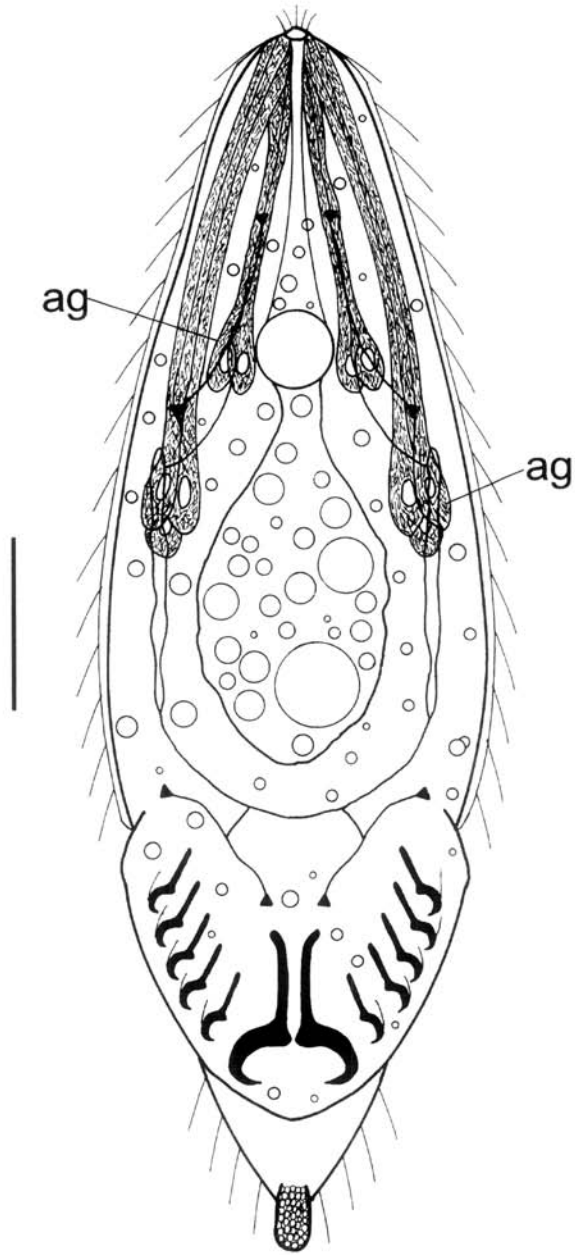

Figure 19 Oncomiracidium of *Grubea cochlear* (Monogenea: Polyopisthocotylea: Mazocraeidae) showing two groups of anterior gland cells (ag) on each side of the larva containing needle-like secretory bodies as observed using phase contrast light microscopy. Scale bar = 50 μm. (Modified from Whittington and Kearn, 1990.)

granular secretion were observed at the haptor margin close to hooklets in the larvae of one of these species, *Hexabothrium appendiculatum*, and strands of material, most likely secreted by the posterior gland cells, were noted in specimens that had attached to glass and Perspex surfaces (Whittington, 1987).

There is a startling lack of information about gland cells in larval polystomatid monogeneans, which is especially surprising because the anatomy and chaetotaxy of numerous species is otherwise well documented (Whittington *et al.*, 2000b). Anterior gland cells are certainly present in the larvae of *Polystoma integerrimum* because Combes (1968) demonstrated that oncomiracidia migrate from the branchial cavity of tadpoles to the bladder via the ventral skin using the anterior glands. Furthermore, he noted that the glands in larval *P. integerrimum* became enlarged and their contents became refringent before migration occurred, indicating an emphasis on their importance during the journey. Observations on the biology of *Protopolystoma xenopodis* by Tinsley and Wynne Owen (1975) suggest that larvae and juveniles can attach and move freely on or in the host toad, *Xenopus*, during migrations via the cloaca, urinary ducts and kidneys to the urinary bladder, but no mention was made of anterior glandular secretions that may be adhesive. The unique invasion route of the desert toad, *Scaphiopus*, by larvae of *Pseudodiplorchis americanus* and *Neodiplorchis scaphiopodis* and described by Tinsley and Earle (1983), would also appear to require looping locomotion, but whether anterior attachment is achieved by suction or by adhesives was not mentioned. Tinsley and Jackson (1986), however, did describe briefly the behaviour of four week old juveniles of *P. americanus* during migration to the bladder via the gut of the toad. Alternate attachment by haptor and 'oral sucker' was mentioned, but again, no reference was made to whether anterior attachment is accomplished by secretions or suction (Tinsley and Jackson, 1986). The oncomiracidia and especially the juvenile, pre-migrant specimens of *P. americanus* are well endowed with gland cells in many regions of the body, but they have not been studied in detail (Richard Tinsley and Jo Cable, peronal communication). Recent studies on the host recognition behaviour of polystomatid larvae by Du Preez *et al.* (1997) also noted that oncomiracidia loop once contact has been made with the surface of a tadpole, but no details were given of the method of anterior attachment.

5.4.2. *Adults*

As mentioned in Section 5.1, the general perception is that adult polyopisthocotyleans in their typical habitat, the gills of fishes, are sedentary. Indeed many families possess an asymmetrical haptor that bears clamps only on one side and previous studies demonstrate that 'right-' or 'left-footed' individuals can occur in the same species. This indicates that asymmetry may depend on

which side of the gills the parasites settle as larvae. This aspect of polyopisthocotyleans is reviewed by Kearn (1994, 1998). Kearn (1998) comments that these asymmetrical worms are most likely sedentary and notes that they 'do not possess conspicuous anterior adhesive glands characteristic of mobile monogeneans'. It is a fact that most taxonomic descriptions of polyopsithocotyleans make no mention of anterior gland cells or ducts that may play a role in adhesion. Llewellyn (1966) suggested that the adoption of blood feeding by polyopisthocotyleans may have contributed to their sessile life style because their food supply is readily accessible and inexhaustible. Polyopisthocotyleans such as microcotylids and gotocotylids (Figure 2D) that have numerous haptoral clamps seem unlikely to have the capacity to coordinate the movement of so many clamps. *Plectanocotyle* (Plectanocotylidae), however, may be mobile (Llewellyn, 1966). Rees (1986) has confirmed that adult *P. gurnardi* are able to move using anterior adhesives, but there is a need for a detailed analysis in this and other adult polyopisthocotyleans to assess the presence of anterior adhesive secretions and to study their ultrastructure.

It is difficult to determine the relative contributions to anterior attachment by suction and by adhesives. An anterior organ that can generate suction is known in many polyopisthocotyleans (Section 5.1), but the roles of secretion and suction in attachment may not be independent for two reasons: 1) a secretion may aid suction by sealing the peripheral rim of a sucker (such as the false oral sucker of some polyopisthocotyleans); 2) suction can be generated by pulling apart two objects separated only by a thin, watery secretion (Nachtigall, 1974). Clearly, a significant amount of investigation is required to determine more about anterior attachment, and especially the role of adhesives or other secretions, in polyopisthocotylean monogeneans.

5.5. Adhesive Secretions on Monogenean Eggs

Section 4.7 highlighted the fact that some temnocephalans attach their eggs by adhesives to the surfaces of their hosts. This strategy has been employed by very few monogeneans because the majority of species lay eggs that are deposited freely into the water column. Fried and Haseeb (1991) commented that 'filaments [of monogenean eggs] presumably have adhesive properties that help attach the eggs to a host or substratum'. Such reports are relatively rare (Kearn, 1986), however, and surface residues that may occur on freshly laid eggs could be remnants of secretions from the process of egg formation. There is a single report of monogenean eggs adhering to host epithelial surfaces. Bychowsky (1957, p. 91, English translation – 1961) noted that eggs of *Nitzschia sturionis* (Capsalidae) were cemented firmly by their appendages to the mucous membrane of the buccal cavity of its sturgeon host. Eggs of another capsalid *Tristomum* (= *Capsala*) *biparasiticum* were reported attached by their

appendages to the ventral surface of copepods found on the gills of tunny (Goto, 1894). Schell (1972) and Byrnes (1986) reported a similar phenomenon for *Udonella* eggs attached to caligid copepods. These adhesives have not been studied, but it would seem that firm attachment of eggs for the duration of embryonation to a hard substrate like an arthropod carapace would place fewer demands on an adhesive than one that must anchor eggs to a secretory surface such as fish epithelium. Other reports of adhesives on monogenean eggs relate to anchoring eggs to a substrate and/or tethering eggs together in bundles. The following species represent some of those reported to lay eggs that bear adhesive material of some sort: *Acanthocotyle lobianchi* (see Kearn, 1967a) (Acanthocotylidae); *Entobdella australis* (see Kearn, 1978) and *E. soleae* (see Kearn, 1963a) (Capsalidae); *Enoplocotyle kidakoi* (see Kearn, 1993) (Enoplocotylidae); *Oogyrodactylus farlowellae* (see Harris, 1983) (Oogyrodactylidae); *Concinnocotyla australensis* (see Whittington and Pichelin, 1991) (Polystomatidae). Kritsky and Boeger (1991) described eggs with 'filament caps' for other oviparous gyrodactylids similar to *O. farlowellae* and it seems likely to us that these are adhesives.

Virtually nothing is known of the nature of the adhesives reported on monogenean eggs. Kearn (1963a) noted that the appendage of *Entobdella soleae* eggs bears between eight and 11 colourless globules approximately 60 μm apart that are insoluble in 4% formalin and in 70% alcohol, even after exposure to these agents for 6 months. The globules are known to be sticky and attach eggs to sand particles (Kearn, 1963b). The adhesive droplets are added in the tubular, proximal region of the ootype (Kearn, 1985) and are derived from one of two types of Mehlis' gland secretion (Tappenden and Kearn, 1999). For *E. australis*, Kearn (1978) observed a single large droplet of adhesive material at the tip of the egg appendage and commented that the source of this adhesive was a large and conspicuous group of lateral gland cells that open into the base of the ootype. The contents of these gland cells and the material known to be sticky on the egg appendage stained similarly with Cason's technique (Kearn, 1978).

5.6. Chemistry of Monogenean Adhesives

Little is published about the chemistry of monogenean adhesive secretions and what is known is based on histochemistry. The anterior head glands of *Leptocotyle minor* (Microbothriidae) stain well with Ehrlich's haematoxylin and with neutral red (Kearn, 1965), those of *Udonella* (Udonellidae) are eosinophilic and granular rather than mucoid (Nichols, 1975) and Lyons (1968) found mucoprotein in anterior secretions of *Entobdella soleae* (Capsalidae). Kritsky (1978), El-Naggar and Serag (1987) and El-Naggar *et al.* (1993) reported staining of all of the different secretion types found in the

anterior head region of a variety of monopisthocotylean monogeneans. All of these authors reported that: rod-shaped bodies (S1 bodies, Table 1) are strongly acidophilic and stain with eosin and light green; spherical secretion (S2 vesicles, Table 1) is 'fairly acidophilic'; when a third anterior secretory type is present (spherical S3 vesicles, Table 1) like in *Gyrodactylus eucaliae* (Gyrodactylidae), they are strongly basophilic (Kritsky, 1978).

There is a similar lack of data for the basic chemical composition of posterior secretions. Kearn (1965) found that the haptoral 'cement' of the microbothriid, *L. minor*, was PAS-negative. Rand *et al.* (1986) summarized histochemical data for the posterior secretions of a different microbothriid, *Dermopthirius carcharhini,* as being a tyrosine-rich lipoprotein. Nichols (1975) commented that the two types of granules in the posterior glands supplying the attachment organ of *Udonella* are eosinophilic.

The anterior adhesive secretions of a single monopisthocotylean monogenean, the capsalid *Entobdella soleae*, have been characterized partially by methods other than histochemistry (Hamwood, Cribb, Halliday, Kearn and Whittington, unpublished data). *In situ* digestion of the rod-shaped bodies in thin sections of adults indicates a proteinaceous composition, at least in part. Proteolytic enzymes digest extruded secretion on glass surfaces in the form of 'pad-prints'. Amino acid analysis of pre-secreted but extracted adhesive material, and also of extruded adhesive material on glass, demonstrates a distinct and repeatable composition that differs significantly from other marine adhesive proteins. Extruded adhesives from a range of species in the Monocotylidae demonstrate the same overall amino acid composition, but with some differences which appear to reflect taxon groupings (Hamwood, Cribb, Halliday, Kearn and Whittington, unpublished data). Currently, evidence for the presence of mucopolysaccharides is equivocal.

5.7. Implications of Adhesives for Parasitism by Monogeneans

A possible relationship between the anterior adhesive areas and host type is not supported at the ultrastructural level in the few monogenean species examined so far. Initial supportive evidence that the ultrastructure of secretory bodies of congeners from the same host species (Whittington and Cribb, 1999) or parasitizing the same host group (Whittington and Cribb, 1998a) are virtually identical is contra-indicated by observations that anterior electron-lucent spherical vesicles observed in some monogenean species from some teleost species (Kritsky, 1978; El-Naggar and Kearn, 1980; Kearn and Evans-Gowing, 1998) are not present in other closely related species also found on teleosts (Whittington and Cribb, 1999). However, differences may relate to individual host species rather than to larger host groupings such as teleosts and elasmobranchs. Previous ultrastructural studies have shown that congeners in the

same microhabitat on the same, or on closely related, host species have similar anterior secretions (El-Naggar and Kearn, 1980; Whittington and Cribb, 1998a). This suggested a possible relationship between secretory types and microhabitat. However we have shown that two congeners from the same host species but from different microhabitats have almost identical anterior secretions (Whittington and Cribb, 1999). Many more species must be examined before possible trends are inferred. Caution is also required, of course, because differences in secretory body appearance do not necessarily imply differences in function or chemistry. There is also a need for studies on the interactions of different anterior secretions because these may indicate some functional similarities.

The anterior adhesive secretions themselves or the anterior attachment areas *in toto* may play a role in host recognition and even in the maintenance of monogeneans on the host (Whittington *et al.*, 2000a). Monogeneans are remarkably host-specific and their specific host species, and also the precise microhabitats on them, are defined structurally, biochemically and physiologically such that they are almost identical in each specimen of a particular host species (Sukhdeo and Bansemir, 1996). Experiments that have examined what may determine host-specificity in monogeneans demonstrate that epidermal mucous cells of specific fish hosts contain or produce an as yet unidentified component implicated in influencing larval attachment (see review in Whittington *et al.*, 2000a). It appears likely that non-host species (i.e. 'alien hosts') may not produce the factor(s) which stimulate attachment by the infective stages of monogeneans, and forced introductions or transplants do not prosper (Kearn, 1967b; Whittington *et al.*, 2000a). Such an epidermal factor (or factors) might act in one of three ways on the parasites: as a chemoattractant; as a signal to chemoreceptors of a favourable site perhaps via nutrients; and/or through the presence of toxic or detrimental components (Buchman, 1998a). Initial attachment by monogenean larvae or newborn gyrodactylid daughters to a host is via anterior attachment using the adhesive secretions and so this region is the probable site through which such a factor or factors may act (Whittington *et al.*, 2000a). Work on gyrodactylids indicates that the anterior attachment region contains mannose-rich glycoproteins which are implicated in stimulating the alternate complement pathway in the host (Buchmann, 1998b, c) and therefore this region is immunologically provocative. On the basis of these data, Whittington *et al.* (2000a) proposed that the anterior attachment region of the monogenean and the surface of the host may contribute to the mediation of the parasite–host relationship. The anterior adhesive secretions themselves, or components of the specialized adhesive area tegument at the site of anterior attachment (e.g. Figure 17), may interact chemically with host epithelium and/or with mucus enabling a 'match'. This may result either in successful establishment of the worm or in a response which may terminate attempted attachment in the

short term (e.g. seconds or minutes) or long term (e.g. days). Specific differences in host fish epithelium and specific differences in monogenean anterior adhesive chemistry (Section 5.6), or in the chemistry of the specialized anterior adhesive area tegument, may all contribute to parasite–host specificity among the Monogenea, and this hypothesis is worthy of further investigation (Whittington *et al.*, 2000a).

5.8. Evolutionary Implications

Unlike Section 4.9 in which phylogenetic considerations from the various studies on turbellarian adhesive systems were discussed, the organs and gland cells that secrete adhesives of too few monogenean species have been examined in detail to permit a similar phylogenetic consideration. A brief discussion of phylogeny based on adhesives across the platyhelminths is presented in Section 10. Whittington and Cribb (1999) pointed out that further comparative study of anterior adhesive areas and their secretions, to embrace different species from more monogenean families, including representatives from the Polyopisthocotylea, may permit the proposal and construction of more meaningful hypotheses on monogenean phylogeny based on a variety of anterior characters.

There is disagreement about phylogenetic relationships within the Monogenea although the class has been investigated extensively. Previous studies have used the haptor and its ontogeny (e.g. Llewellyn, 1963, 1970), larval sensilla and chaetotaxy (Lambert, 1980a, b), ultrastructure of sperm and spermiogenesis (e.g. Justine *et al.*, 1985; Justine, 1991) and larval and adult morphology but also incorporating sperm ultrastructure (e.g. Boeger and Kritsky, 1993, 1997, in press). It was anticipated that molecular analyses may resolve controversial relationships, but this was not the case with earlier studies (e.g. Blair, 1993; Rohde *et al.*, 1993; Mollaret *et al.*, 1997). It is apparent that robust reconstruction of phylogeny will not yield to single techniques and therefore many approaches are necessary like the 'total evidence' approach incorporating information from morphology and molecules proposed by Littlewood *et al.* (1999). To resolve phylogeny further among the Platyhelminthes, additional morphological data together with molecular data from more taxa were suggested by Littlewood *et al.* (1999).

For the Monogenea, it is noteworthy that many taxonomic descriptions pay little attention to details of the anterior regions. Despite the diversity known, few studies have used these anterior characters in systematic analyses (e.g. Whittington and Horton, 1996; Chisholm and Whittington, 1996b, 1997; Whittington *et al.*, in press). Indeed, a recent phylogeny and classification of Monogenea by Boeger and Kritsky (1997) used 38 characters including seven haptoral and 12 spermatozoa and spermiogenesis

characters, but only a single character related to the anterior end was used (Whittington and Cribb, 1999). Furthermore, the anterior character that was incorporated into Boeger and Kritsky's (1997) phylogeny was the 'circumoral sucker', an organ *sensu stricto* that, according to some studies, may not be present in any monogenean (e.g. Whittington *et al.*, 1989, and Section 5.1, p. 148)! It is apparent, therefore, that a wealth of potentially useful information about the anterior region of the Monogenea has been ignored to date. Our investigations, and many of the studies reviewed above, demonstrate that a variety of anterior adhesive structures and secretions exist. We think that future attention paid to these characters will provide useful additional morphological data of the kind suggested by Littlewood *et al.* (1999) to add to the 'total evidence' approach to help assess phylogeny for the Monogenea. We have pointed out (Whittington and Cribb, 1999) that our future studies will continue to investigate not only the morphology and ultrastructure of the anterior adhesive areas across the Monogenea but will also examine the chemistry of the different secretory types (e.g. Hamwood, Cribb, Halliday, Kearn and Whittington, unpublished data) to incorporate into phylogenetic assessments.

Opinions vary on the origins and evolution of the parasitic platyhelminths but Jennings (1997) considered that adhesive glandular secretions and suckers in ancestral turbellarians, which are also widespread in extant free-living turbellarians, may have been critical pre-adaptations that favoured symbiosis. Similarly, adhesive glandular secretions in ancestral monogeneans are likely to have represented an important pre-adaptation to their future ectoparasitic lifestyle and Kearn (1994, 1998) has described at length how the Monogenea may have diverged on their fish and aquatic and semi-aquatic tetrapod hosts. The continual development and evolution of effective anterior adhesives by the Monogenea, especially among the monopisthocotyleans, has played an important role in their life as ectoparasites of, principally, fishes. Kearn (1994) has suggested that although locomotion may be costly in terms of the energy budget of the worms and also in terms of increasing the chances of dislodgement during movement, a particular advantage of mobility is to promote cross-insemination between conspecifics and therefore maintain genetic flexibility. Another advantage for mobility in monogeneans is that regular changes in location may reduce or even prevent the onset of a local inflammatory response by the host mounted against the parasite (Kearn, 1994, 1998). Whittington *et al.* (2000a) have proposed another advantage of mobility among the Monogenea by suggesting a role for adhesives, or the anterior region through which the adhesives are secreted, in host specificity (Section 5.7).

The importance of locomotion and mobility in the Monogenea, especially among the monopisthocotyleans, may be reflected by the likelihood that adhesive secretions, especially those released by the haptor, have evolved

independently, perhaps on several occasions, in the class. Microbothriids and udonellids attach to hard surfaces using posterior secretions and *Anoplodiscus* (Anoplodiscidae) similarly attaches to fin rays (Section 5.3). Further study may demonstrate whether there is a phylogenetic link between these families based on their posterior adhesives. Haptoral adhesives among dactylogyrids such as *Chauhanellus* and *Hamatopeduncularia* may have a separate origin from those in *Neocalceostomatoides* (see Kearn, 1998). These studies, together with further studies of morphology, ultrastructure and chemistry of the anterior secretions of monogeneans (see above), seem likely to be phylogenetically informative.

5.9. Summary

Considering that the leech-like looping locomotion of monogeneans is well known and seemingly of central importance to their biology, it is surprising that their remarkable adhesive secretions, especially those released by the anterior gland cells, have received so little attention until relatively recently. Unlike the turbellarians for which the adhesive systems of numerous species representing several higher taxa have been studied, analysis of the anterior adhesive systems of monogeneans is in its infancy, with only 15 species from five families studied in detail (Table 1). Further investigation not only has implications for phylogeny, but may shed light on the mechanism and possible interactions between different secretory types that may elicit the instant, but strong, adhesive bond between parasite and host epithelium/mucus. There is an indication that instant detachment of the parasites from the host is achieved by the specialized adhesive area tegument (Kearn and Evans-Gowing, 1998) and more research may shed further light on the mechanism(s) involved. Attachment using bioadhesives to live surfaces has received virtually no attention. We have introduced a new term 'tissue adhesion' (Section 3.4) to distinguish and highlight the importance of, and the differences between, adhesion displayed by, for example, monopisthocotylean monogeneans and adhesion by other marine invertebrates that attach to abiotic substrates. Further research, especially on adhesive chemistry, may lead to products of commercial significance. These may include development of instantly reversible adhesives capable of operation in moist or wet environments, even underwater, or adhesives that can bind to epithelial surfaces. Such adhesives may have significance for surgery, dentistry and research procedures. An understanding of how monogenean adhesives operate may also help to *prevent* their function and could, therefore, provide a possible control measure for monogeneans that are pathogenic in aquaculture.

6. ADHESIVE AND OTHER GLAND CELLS IN THE CESTODES

Here the cestodes include the eucestodes, gyrocotylids and amphilinids following Rohde (1994b) and we treat each group separately. To the lay person, the principal attachment organ of the true tapeworms (= Eucestoda), the anterior scolex (e.g. Figure 3A), is probably the most quintessential of parasite holdfasts. The scolex can be provided with shallow grooves, suckers of various kinds, hooks, spines, tentacles and glands or combinations of these, but it can also be simple or absent. The mode of attachment by adult tapeworms from several orders has been reported (e.g. Pseudophyllidea: see Hogans and Hurley, 1986; Tetraphyllidea: see Borucinska and Caira, 1993; McKenzie and Caira, 1998; Trypanorhyncha: see Borucinska and Caira, 1993; Campbell and Callahan, 1998). Coil (1991) is recommended for a broad coverage of mechanical attachment by cestodes. As for many endoparasites, it was long thought that tapeworms had neither the capacity nor the need to move inside their hosts. Studies 60 years ago, however, indicated that there was some migration inside the vertebrate gut by *Hymenolepis diminuta* (Cyclophyllidea) and locomotory movements are also known for *Diphyllobothrium* (Cyclophyllidea) (see Kearn, 1998). Anterior progress in *H. diminuta* is made using pairs of suckers pushed forwards in a stepwise manner (Kearn, 1998) and this is probably assisted by anteroposterior peristaltic contractions of the strobila against the gut contents (Sukhdeo and Kerr, 1992). Interestingly, Wardle and McLeod (1952) considered that the bothridia of tetraphyllids were 'essentially organs of locomotion rather than of fixation ...'.

There are many reports of gland cells in the scolices of several tapeworm orders (e.g. Stoitsova *et al.*, 1997), but their role in adults is considered enigmatic (Roberts and Janovy, 1996). Depending on the cestode group, the complexity of the life cycle confers differing demands on the infective stages and there are anterior gland cells reported in the lycophore, coracidia and hexacanth larvae and in metacestode stages. Knowledge of a variety of gland cells and of their secretions in cestodes, only some of which may have an adhesion function, are reviewed below.

6.1. Larvae and Metacestodes of Eucestoda

Unlike the miracidia and cercariae of some digeneans that may locate the next host in the life cycle actively, the ciliated coracidia of pseudophyllideans and some tetraphyllideans that hatch actively from eggs are then consumed by the next, usually arthropod, host (Kearn, 1998). Penetration of the arthropod gut is achieved using the six hooks of the infective stage (e.g. Kearn, 1998), but it is possible that histolytic glandular secretions are also involved. There is evidence

that this is the case at least in the oncospheres of *D. latum*, *Bothriocephalus gowkongensis* (see Kuperman and Davydov, 1982a) and *Triaenophorus nodulosus* (Pseudophyllidea) (see Korneva, 1994; Davydov and Korneva, 1997). For the cyclophyllideans, the encapsulated hexacanth larva emerges from its tiny egg and similarly penetrates the gut wall of the intermediate host. Kashin (1986) identified acid and neutral mucopolysaccharides in the 'penetration glands' from oncospheres of nine species of Dilepididae and ten species of Hymenolepididae. Evidence for the role of histolytic secretions to assist penetration of the beetle gut by the hexacanth of *Hymenolepis diminuta* is provided by Moczon (1977, 1996a); the nature of the penetration glands was determined by histochemistry to be a serine protease (Moczon, 1996a). Penetration glands are also known for *Echinococcus granulosus* (Cyclophyllidea), but the same secretions are also thought to protect the parasite from the host immune response (Holcman and Heath, 1996). Kashin (1986) made a similar suggestion for a coating of polysaccharides on the surface of dilepidid and hymenolepidid oncospheres in the coelomic cavity of their invertebrate hosts. He has suggested that the mucopolysaccharides secreted by the 'penetration glands' of some dilepidid and hymenolepidid oncospheres may also participate in adhesion during their migration inside the intermediate host.

Gland cells are not only restricted to larvae of eucestodes because frontal glands are known in many metacestodes. For example, anterior or frontal glands are reported from: procercoids of *Haplobothrium globuliforme* (Haplobothrioidea) (see MacKinnon *et al.*, 1985), *Caryophyllaeus laticeps*, *Khawia armeniaca*, *K. sinensis*, *Archigetes sieboldi* (Caryophyllidea) (see Davydov and Poddubnaya, 1988), *Eubothrium rugosum*, *Bothriocephalus gowkongensis* and *Triaenophorus nodulosus* (Pseudophyllidea) (see Kuperman and Davydov, 1982a; Davydov and Korneva, 1997); unencapsulated plerocercoids of *D. latum*, *T. crassus* (see Davydov, 1981), *E. rugosum* (see Kuperman and Davydov, 1982a), *Diphyllobothrium* sp. ind. (see Yazaki *et al.*, 1985) and *Spirometra erinaceieuropaei* (see Okino and Hatsushika, 1996); encapsulated plerocercoids of *T. nodulosus* (Pseudophyllidea) (see Davydov and Korneva, 1997). There appears to be a general concensus that the frontal or anterior glands in metacestodes are for penetration. Indeed Davydov (1981) performed experiments by introducing plerocercoids of *D. latum* removed from muscle tissue of a variety of fishes into the stomach of a recipient fish. He observed these plerocercoids in the process of penetrating the stomach wall and their journey ended in the body cavity or muscle. Kuperman and Davydov (1982a) reported that the amount of secretion in glands of plerocercoids that had passed through the stomach wall was significantly decreased. Polzer *et al.* (1994) demonstrated proteinase activity in the plerocercoid of *Proteocephalus ambloplitis* (Proteocephalidea) and suggested that it was secreted from the 'apical organ'.

It seems to us unlikely that most metacestode stages would require any adhesive secretions or an attachment mechanism of any kind when their biology is considered. Most procercoids and plerocercoids reside in the body cavity of their hosts, they may be encapsulated, encysted or neither, and they have no need for an attachment mechanism because they generally lie loose in the capsule or cyst, or in or on the viscera. Their requirement for movement is necessary only when they, together with their host, are eaten by a subsequent host. The only exceptions to this general scheme are the plerocercoids of some tetraphyllideans, or so-called 'scolex polymorphus', many of which live actively, moving using their anterior suckers, in the intestines of their squid (Stunkard, 1977) and teleost hosts (unpublished observation, I.D. Whittington). These plerocercoids are similarly awaiting ingestion by the elasmobranch definitive host.

Ultrastructure of glands in immature or juvenile eucestodes (oncospheres, procercoids and plerocercoids) from the Pseudophyllidea show electron-dense, round secretory granules which may vary in size between stages or may be consistent in appearance (Kuperman and Davydov, 1982a). Size also varies across species. In plerocercoids of *Eubothrium* and *Triaenophorus*, a double membrane surrounds the secretory granules. Microtubules are usually present in the duct walls. The general scheme of secretion involves the secretory material passing into the tegument where, together with the tegumental cytoplasm, it forms a surface evagination through which the secretion is released (Kuperman and Davydov, 1982a). The secretion accumulates in the evaginations in species of *Eubothrium* and *Triaenophorus* plerocercoids. However in *Diphyllobothrium* species, the granules do not accumulate and the ducts open directly onto the body surface (Kuperman and Davydov, 1982a).

A detailed ultrastructural study, including some histochemistry, was made by Brockerhoff and Jones (1995) of the scolex and tentacles of the metacestode of a *Polypocephalus* species (Lecanicephalidae) from crab (Decapoda) musculature. This unencapsulated metacestode (M.K. Jones, personal communication) lacked mechanical armature but possessed a pars apicalis comprising approximately 400 tentacles, a scolex cavity containing glandular tissue and posteriorly, a large scolex gland (Brockerhoff and Jones, 1995). Electron-dense, round vesicles produced by tissue of the scolex gland and scolex cavity fill the tegument of the tentacles and pars apicalis which is typically syncytial (Figure 20). Histochemical studies revealed proteins and neutral mucosubstances in the tentacles, scolex gland and scolex cavity indicating a glycoprotein secretion to which Brockerhoff and Jones (1995) ascribed an adhesive role. No evidence of exocytosis was found in this metacestode and it is presumed that the glands are functional only in the adult (M.K. Jones, personal communication). Further details of attachment by adult lecanicephalids are presented in the next section.

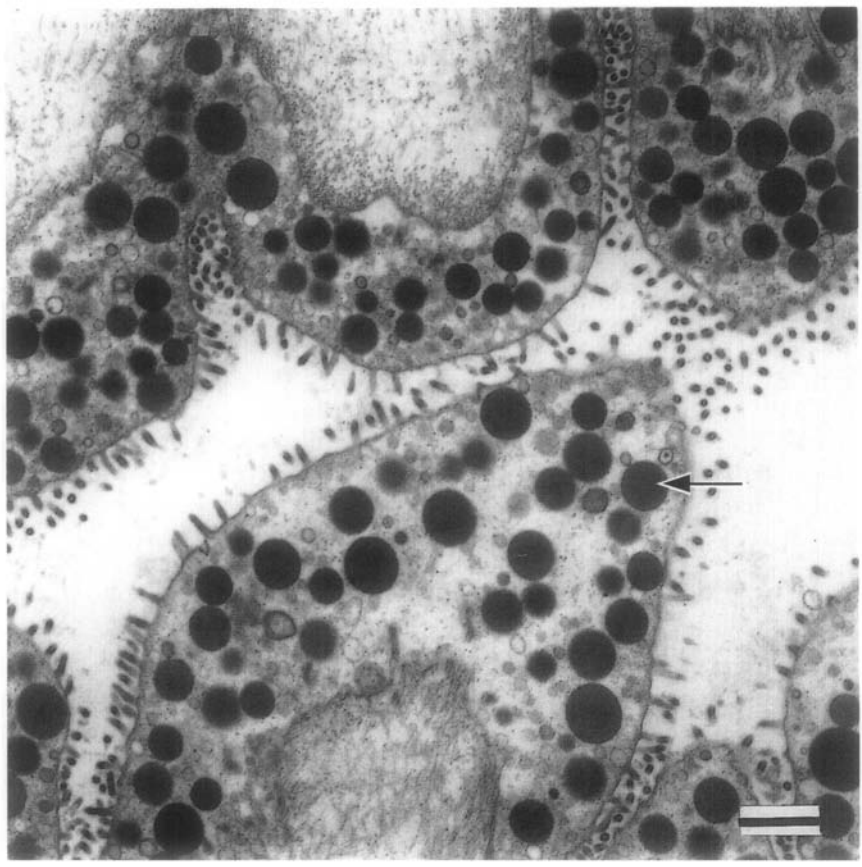

Figure 20 Transmission electron micrograph of the tentacles of an unencapsulated metacestode of a *Polypocephalus* sp. (Cestoda: Lecanicephalidea) from the musculature of a crab. Note the secretory tegument of the tentacles (arrow indicates electron dense secretory body). Scale bar = 1 μm. (Dr M.K. Jones, original and unpublished photograph.)

6.2. Adult Eucestoda

In a comprehensive treatment of the biology of parasitism in the flatworms, Kearn (1998) drew attention to the work of Andersen (1975) on *Diphyllobothrium latum* and *D. ditremum* (Pseudophyllidea) and the observation that a layer of material, which Kearn suggested may be cement, occurred between the surface of the bothria and the lining of the intestine of their human host. Kearn also urged further investigation of attachment in tetraphyllideans and suggested that use of cement may be a possibility for anchoring the thin, leaf-like suckers of *Phyllobothrium pirei* to the villi of their elasmobranch

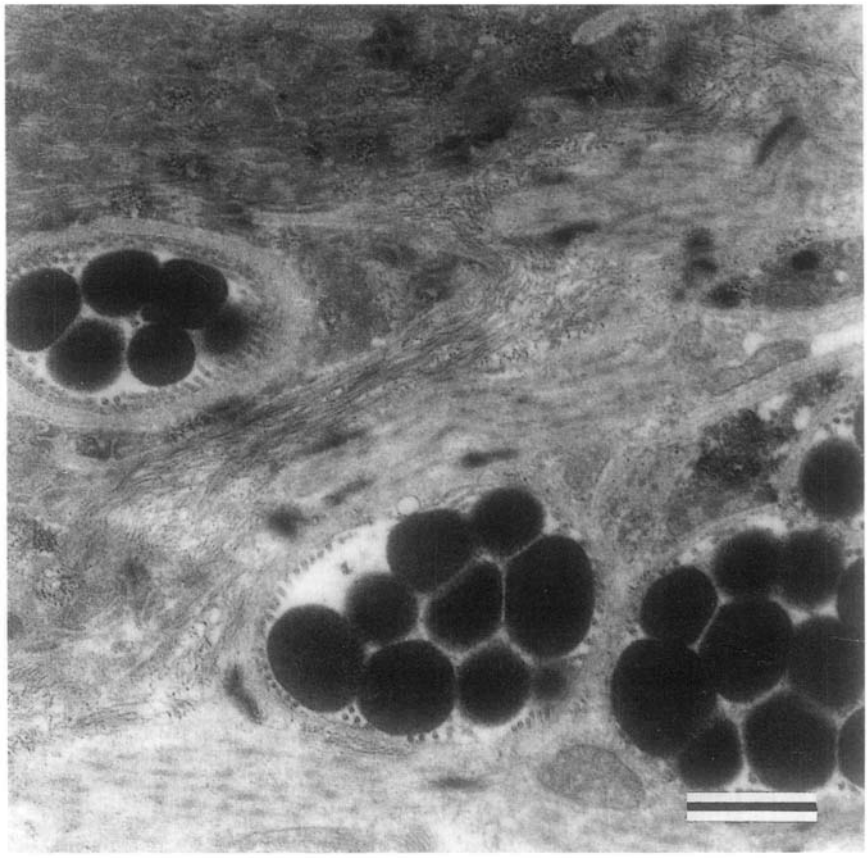

Figure 21 Transmission electron micrograph of the frontal gland secretion (eccrine glands according to Jones and Beveridge, 1998) from *Nybelinia queenslandensis* (Cestoda: Trypanorhyncha) from the gut of *Carcharinus melanopterus* (Carcharinidae). Note the multiple rounded secretory bodies in each duct and the microtubules lining the duct walls. Scale bar = 500 nm. (Dr M.K. Jones, original and unpublished photograph.)

host. Butler (1987) remarked that the scolex of the tetraphyllid *Rhinebothrium pearsoni* was active and could attach to glass surfaces, but no mention was made of whether attachment was secured by suction, secretion or perhaps by both. There are many reports among adult tapeworms of 'scolex gland cells' or 'scolex glands' (e.g. Hayunga, 1979a; McCullough and Fairweather, 1989; Stoitsova *et al.*, 1997; Zd'arska and Nebesarova, 1997, 1999), 'glandular apparatus' with ducts leading anteriorly (e.g. Timoshechkina, 1984), 'frontal glands' (e.g. Davydov and Biserova, 1985; Davydov and Mikryakov, 1986; Farooqi, 1986; Davydov and Poddubnaya, 1988; Okino and Hatsushika, 1996;

Jones and Beveridge, 1998) (Figure 21) and 'rostellar glandular apparatus' (e.g. Pospekhova *et al.*, 1988). Gland localization in most adult cestodes is in the scolex, although in some taxa, glands are found in the anterior and middle part of the body (Kuperman and Davydov, 1982b).

The secretory products of the gland cells can be discharged in three different ways. In some members of the Pseudophyllidea, the electron-dense membrane bound secretion (rounded or various shapes) travels through ducts and into the tegument where, together with the tegumental cytoplasm, they form projections for secretion (the apocrine mechanism; Kuperman and Davydov, 1982b). Members of the Cyclophyllidea also show the apocrine mechanism (Stoitsova *et al.*, 1997). In others such as *Diphyllobothrium latum*, discharge is via ducts that travel through the tegument (eccrine secretion; Kuperman and Davydov, 1982b). The frontal glands of *Nybelinia queenslandensis* (Trypanorhyncha) described by Jones and Beveridge (1998) also appear to be eccrine glands (Figure 21). This is also seen in the Tetraphyllidea (see McCullough and Fairweather, 1989). In some members of the Caryophyllidea, microapocrine secretion is seen where small evaginations of the tegument, containing electron-dense secretory granules, detach (Kuperman and Davydov, 1982b). Usually the vesicles are electron-dense but in *Hunterella nodulosa* the vesicles are electron-lucent (Hayunga, 1979a). Mostly the secretory granules are reported as rounded (e.g. Figure 21) but in the Proteocephalidea they appear as elongated ovals or rod-shaped (Kuperman and Davydov, 1982b). Several functions for the anterior glands in adult tapeworms have been proposed and these are considered below.

Investigations of the anterior glands in adult cestodes have been histological (e.g. Farooqi, 1986; Yazaki *et al.*, 1985), ultrastructural (e.g. Hayunga, 1979a; Kuperman and Davydov, 1982b; Timoshechkina, 1984; Zd'arska and Nebesarova, 1997, 1999) and a few also include some histochemistry and cytochemistry (e.g. Hayunga, 1979a; McCullough and Fairweather, 1989; Stoitsova *et al.*, 1997). Likely function(s) is based on supposition, and whether or not there is correspondence between the origin and role of anterior glands in larval, metacestode and adult tapeworms remains to be demonstrated (Section 10). Farooqi (1986) reported that frontal glands in juveniles and metacestodes of *Tentacularia coryphaenae* (Trypanorhyncha) persisted in adult stages, but that their prolific development in the juvenile stage suggested a role of importance for the establishment of the parasite in the host. He proposed a histolytic function to assist tentacle penetration, a function to protect the worm from digestion by the host and perhaps bestow some antigenic mimicry to the parasites (Farooqi, 1986). A role for anterior glandular secretions from adults in defence or protection from the host's immune system has also been suggested by Timoshechkina (1984), Davydov and Mikryakov (1988) and Pospekhova *et al.* (1988). Histochemistry of the anterior secretions from adults has revealed lipoprotein indicative of immunological importance and acid mucopolysaccharides and

protein suggestive of a role in defence (Kuperman and Davydov, 1982b). The demonstration of glycoprotein in some has led to suggestions for proteolysis, adhesion and protection (Stoitsova *et al.*, 1997). Another function considered includes a role in strobilization (McCullough and Fairweather, 1989). Zd'arska and Nebesarova (1999) made the interesting suggestion that secretions produced by the most anterior glands in *Proteocephalus macrocephalus* (Proteocephalidea) may either be adhesive or may isolate the anterior part of the parasite from the intestinal tissue to decrease any immune response. Yazaki *et al.* (1985) studied recently established specimens of *Diphyllobothrium* sp. ind. and noted that secretion from the 'green gland' (when stained with PAF) may assist attachment of the bothrial lobes.

Hayunga (1979a, b) and McCullough and Fairweather (1989) provide the most direct evidence for use of an adhesive by adult cestodes. Scolex glands were identified in adult specimens of *Hunterella nodulosa*, but not in *Glaridacris laruei* or *G. catostomi* (Caryophyllidea) (see Hayunga, 1979a). Histochemical tests demonstrated that the contents of the scolex glands of *H. nodulosa* are active in protein synthesis and secretion occurred intrategumentally and not via ducts and pores. Electron lucent vesicles pass along cytoplasmic processes from the gland cells into the syncytial tegument and fuse with the surface membrane to release their contents (Hayunga, 1979a). Studies at the parasite–host interface indicated that the scolex of *H. nodulosa* is separated from host mucosa by an 'eosinophilic matrix' and it is noteworthy that Hayunga (1979a) draws attention to the fact that frontal glands seem to be more numerous in caryophyllideans that have poorly developed mechanical attachment apparatus. It is also interesting that Hayunga (1979b) noted considerable damage at the attachment site of *H. nodulosa* to the intestine of its host fish and speculated that the adhesive secretion may also be an irritant that provokes the inflammatory response observed. A glycoprotein secretion from the syncytial scolex gland of *Trilocularia acanthiaevulgaris* (Tetraphyllidea) was proposed by McCullough and Fairweather (1989) as an adhesive to aid attachment to host mucosa. It was suggested that the adhesive may be more important in juvenile worms in the process of establishing themselves in the definitive host during the early stages of infection because the scolex of these specimens is less well developed.

Stoitsova *et al.* (1997) highlighted the fact that 'scolex glands' are reported widely among adult cestodes, but pointed out that most studies had examined tapeworms with additional attachment mechanisms. The inference is that cestodes without an elaborate scolex may rely more on alternative methods of attachment, perhaps by adhesion. McCullough and Fairweather (1989) reviewed the structure and chemistry of scolex glands, but as they emphasized, there is no definitive biochemical evidence to support an adhesive role. Similarly, there are no direct histological or ultrastructural data that demonstrate an adhesive layer between parasite and host.

Lecanicephalids differ from most other cestodes of elasmobranchs by possessing a bipartite scolex comprising an often elaborate apical holdfast, the pars apicalis, and the pars basalis bearing four suckers (Euzet, 1994). In *Polypocephalus* species, the pars apicalis is considered to be the principal holdfast (Butler, 1987). Tentacles of adults are reported to be embedded deeply into the host's gut mucosa (Butler, 1987), but Brockerhoff and Jones (1995) commented that the mechanism of tentacle penetration and attachment was unknown. The ultrastructural and histochemical study of the scolex and tentacles of a *Polypocephalus* species metacestode (Section 6.1, p. 177) prompted Brockerhoff and Jones to suggest that adults may attach to the mucosa of their elasmobranch definitive hosts using a glycoproteinaceous secretion they assumed to be an adhesive. This seems likely in view of the increased surface area of the pars apicalis, the fact that tentacles appear to interdigitate with host tissue and the lack of any mechanical attachment apparatus. Confirmation of an adhesive secretion in lecanicephalids, however, awaits further study of the closely opposed interfaces between attached adult parasite and host mucosa and further characterization of the secreted material (Brockerhoff and Jones, 1995).

6.3. Gyrocotylidea

The position of gyrocotylideans within the platyhelminths remains hotly debated. Whatever their placement, gyrocotylideans appear to have affinities with amphilinids and cestodes (e.g. Rohde, 1994b) and with cestodes, amphilinids and monogeneans (e.g. Kearn, 1998). They are a small, enigmatic group and extant members inhabit the intestines of holocephalan fish. The life cycle of gyrocotylideans is unresolved but there is evidence that it may be indirect (Rohde, 1994c), although there is certainly scope to interpret current evidence as indicative that it is direct (Kearn, 1998). Eggs of *Gyrocotyle* hatch to release a ciliated, free-swimming stage called a lycophore larva that resembles the oncomiracidium of monogeneans (e.g. Figures 14, 19), having ten hooklets at the posterior end and prominent gland cells at the anterior end (Xylander, 1990; Rohde, 1994c). The presence of anterior gland cells in the lycophore, like those known in monogenean oncomiracidia, indicates a possible role in adhesion, but studies by Manter (1951) suggested a function for tissue penetration.

Xylander (1990) described four pairs of different gland cells in the lycophore of *G. urna*. The gland cell bodies reside at a level about two-thirds of the distance from the anterior end and open via long gland ducts lined by microtubules at the anterior end (Xylander, 1990). Each of the four gland cell types is distinguished by their characteristic membrane-bound secretory bodies (Xylander, 1990). One pair of gland cells, which are uninucleate, with their

ducts located just ventral to the 'brain', contain so-called 'striped' ovoid, moderately electron dense granules ('striped vesicles' or SV in fig. 1 of Xylander, 1990), approximately 340 nm long by 170 nm wide and the striation periodicity is 40 nm. The second pair of gland cells, which are multinucleate, with ducts ventral to the 'brain', contain ovoid secretory bodies, about 230 nm in diameter, with amorphous contents ('vesicles with amorphous content' or GV in figure 1 of Xylander, 1990). The third pair of gland cells are multinucleate, with ducts most dorsal in the body according to Xylander (1990), containing secretory bodies with homogeneous, rounded, moderately electron-dense ultrastructure with a mean diameter of 500 nm (larger vesicles with electron-dense granules or LDV in fig. 1 of Xylander, 1990). Ducts from the fourth pair of uninucleate gland cells are just dorsal to the 'brain' and contain secretory bodies with homogeneous, rounded, moderately electron dense granules with a mean diameter of 500 nm (smaller vesicles with electron-dense granules or SDV in fig. 1 of Xylander, 1990). The three gland cell types described for the lycophore of *Austramphilina elongata* by Rohde (1986, 1987b, and Section 6.4 below) were considered by Xylander (1990) to correspond to three of the gland cell pairs in *G. urna*. We consider, however, that such comparison is premature until more information is available on the histochemistry, chemical nature and possible functions of these gland cells and their products. Comparisons seem especially ill advised until more is revealed about the life cycle of gyrocotylideans and the tasks required of the lycophore of *Gyrocotyle*. Further studies on the infection dynamics and life cycle of *Gyrocotyle* are needed, but it seems likely that conspicuous gland cells at the anterior end of lycophore larvae have discrete roles which may include adhesion to host surfaces as well as penetration, if indeed this process is required.

The posterior end, known as the rosette organ, of adult *Gyrocotyle* does resemble the haptor of several groups of monogeneans. The rosette organ comprises numerous thin folds of tissue that envelop a funnel-shaped invagination that may still bear posterior hooklets dorsally. Lyons (1969) determined that the inner surfaces of the rosette are richly supplied by flask-shaped ventral gland cells that secrete PAS-positive material considered to be an adhesive mucoprotein. Allison (1980) supported an adhesive role for these products and for the entire organ, and she described the extensile nature of the rosette folds and the fact that they can attach firmly to host villi by an adhesive secretion. Kearn (1998) drew comparison between the thin, extensile tissues of the gyrocotylidean rosette organ and the membranous tissues of the haptor of the gill-parasitic monogenean, *Neocalceostomatoides brisbanensis* (Dactylogyridae), another flatworm that can attach securely by the posterior end using an adhesive (Kearn *et al.*, 1995, and Section 5.3.2). Other than the study by Lyons (1969), no details are known of the adhesive secretions in adult *Gyrocotyle*.

6.4. Amphilinidea

Just as the position of the gyrocotylideans within the flatworms is argued, the same is the case for the Amphilinidea. Rohde (1994c) considered that they and the gyrocotylideans are closely related to the eucestodes. For two schools of thought on this topic, see Kearn (1998). Amphilinideans are another small group of flatworms, adults of which occupy the body cavity of fish and turtles. Unlike the gyrocotylideans, their life cycle is known (e.g. Rohde and Georgi, 1983; Rohde, 1994c). Crustaceans are the intermediate hosts and these must be consumed by the definitive vertebrate host to enable the life cycle to be completed.

The ciliated, swimming larva, called a decacanth or lycophore, is provided with ten posterior hooks and three types of frontal or anterior glands (Rohde, 1986). We detail the anterior secretions of *Austramphilina elongata* because of their uniqueness, and for comparison with the anterior secretions of other flatworms. However, there is no evidence that these secretions play a role in adhesion. The lycophore of *A. elongata* was studied using light microscopy and TEM by Rohde (1986). Eleven or 12 gland cells termed type-I containing electron-dense, irregularly round to oval secretory granules lie dorsally in the posterior half of the body. Each cell possesses a duct opening into an anterior tegumental invagination but is covered by epidermis and does not seem to open to the environment. Ten type-II gland cells, positioned ventral to type-I gland cells, contain what Rohde (1986) termed 'long secretory granules', round to irregularly oval in cross-section, and containing longitudinal microtubules within the secretory granules. Ducts of type-II cells open on a single midventral papilla some distance from the anterior end of the body. Ten type-III gland cells lie ventral and anterior to type-I and type-II gland cells and secrete irregular round to oval granules containing, along some of their length, coiled microtubules with an electron dense core. Ducts from type-III cells open at a group of five endings on each side of the body near the anterior end, posterior to the duct openings of type-I cells and anterior to the midventral papilla on which ducts of type-II cells open. Ducts of each gland cell type possess peripheral microtubules and mitochondria. Rohde (1987b) emphasized that the secretory bodies produced by the type-II and type-III cells of *A. elongata* are unique among the parasitic Platyhelminthes because of the presence of microtubules *inside* the secretory products. The secretory bodies of type-I cells are produced by numerous Golgi complexes; type-II cells are formed by Golgi complexes and microtubules seem to condense in the cytoplasm of the cell or inside the granules themselves; type-III cells develop by secretion from Golgi complexes and then microtubules aggregate around and migrate into the granules (Rohde, 1987b).

It is generally assumed that the highly developed anterior glands of larval amphilinideans are for penetration (e.g. Coil, 1991) and Rohde and Georgi (1983), Rohde (1986, 1987b) and Rohde and Watson (1989) should be consulted for observations and studies on this. The possibility exists, however, that

perhaps one of the three secretory types in *A. elongata* may be involved in adhesion as the lycophore travels through the tissues of the crayfish.

Adult amphilinideans live inside the body cavity of their hosts. There is never any mention of attachment by adults inside their hosts and the general impression is that they move about among the viscera. Wardle and McLeod (1952) describe a small, proboscis-like structure that can protrude and retract from the anterior end, is provided with a serrated 'cuticle' and is operated by a massive longitudinal muscle bundle. They considered that this musculature could assist in pulling the rest of the body through perforations made by the proboscis and made the suggestion (or observation?) that amphilinideans could tunnel through the host's body wall to the outside (Wardle and McLeod, 1952). This life style indicates that there may be no requirement for organs of attachment or gland systems that may produce adhesives. Despite this, for the sake of completeness, the few studies of anterior secretions in adult amphilinideans are covered briefly below.

No mention was made by Wardle and McLeod (1952) of anterior gland cells other than their comment that what others had termed frontal glands they considered to be muscle fibres. Nevertheless, frontal glands in adult *Amphilina foliacea* from the body cavity of sturgeon, *Acipenser ruthenus*, were identified by Popova and Davydov (1988). They described two gland cell types: one type produces rounded, electron-dense granules 0.2 to 0.4 μm in diameter; the second type forms granules of varying size (0.2 to 0.8 μm) and shape, but can form aggregations 2 to 2.5 μm long (Popova and Davydov, 1988). They observed that the anterior extremity of the worm formed a cavity when the proboscis was invaginated and that this cavity was always filled with secretion of the second type; the smaller, granular bodies retained their shape after secretion (Popova and Davydov, 1988). These authors drew attention to the fact that frontal secretions in adult *Amphilina foliacea* differed from frontal secretions of the lycophore of *Austramphilina elongata* (see above).

Anterior secretions in adult amphilinideans are thought to play a role in penetration. Popova and Davydov (1988) considered that tissue penetration in *Amphilina foliacea* occurred by a combination of the histolytic properties of the frontal glands and the mechanical action of the muscular proboscis, the surface of which is provided with special spines rather than microvilli. Another secretion described by Popova and Davydov (1988) containing glycosaminoglycans from the tegument of *A. foliacea* was thought to defend the parasite from host encapsulation reactions.

6.5. Summary

Cestodes, or at least the true tapeworms (= Eucestoda), are particularly well known for the diversity of their attachment organs. The tiny scolex, usually

provided with muscular grooves, powerful suckers, hooks and spines or combinations of these, is a highly evolved mechanical attachment device capable of supporting a large body mass. Reports in true tapeworms of anterior secretions that may be adhesive to supplement mechanical attachment may come as a surprise. However the review above indicates that there is some evidence, albeit largely circumstantial, that a variety of glands in many stages of the life cycle may have an adhesive function. That there is considerably less reliance on adhesives in the endoparasitic cestodes than in the free-living and symbiotic turbellarians and in the ectoparasitic monogeneans is not surprising when the mobility of these groups is considered. Some tapeworms can migrate along the lining of the gut, but it is unknown how widespread this behaviour is. A comparison of Section 6 above with Section 7 below suggests that there are more reports of adhesives in cestodes than in digeneans, but this probably reflects a lack of attention paid to mechanisms of supplementary attachment in flukes. Reports of likely adhesive secretions among cestodes and especially in gyrocotylideans, however, may be indicative of a closer phylogenetic relationship between cestodes and monogeneans which, as Section 5 demonstrates, use adhesives widely. Kearn (1998) should be consulted further for a traditional view of possible interrelationships between platyhelminth groups based on an organismal–systematic approach that highlights biological affinities between monogeneans, gyrocotylideans and cestodes.

McCullough and Fairweather (1989) highlighted the prominent development of scolex glands in adult cestodes and the presence of glands in other development stages and suggested that further research may resolve the dual conundrum of identity and function. Nearly 10 years later, Stoitsova *et al.* (1997), in their study of scolex glands in three species of dilepidids (Cyclophyllidea), commented that the function of these gland cells was unresolved. Perhaps research during the next 10 years will reveal their function(s).

7. ADHESIVE AND OTHER GLAND CELLS IN THE DIGENEA

The principal attachment organs in adult digeneans are the muscular suckers (Figure 3B). There is usually, but not always, a powerful oral sucker surrounding the mouth and most also have a ventral sucker, the position of which can be variable depending on the taxon. The functional morphology of suckers has been determined (e.g. Halton, 1967; Smyth and Halton, 1983) and movement like a leech by alternately attaching one or other sucker is known (Fairweather *et al.*, 1983; Sukhdeo and Mettrick, 1986; Sukhdeo *et al.*, 1988; Basch, 1990). There are cases of additional mechanical specializations for attachment among adult digeneans in some families, but reports of mechanical attachment supplemented by adhesives appear to be rare. A few reports exist of

attachment by adhesives in some miracidia and cercariae during their invasion period. The following sections review briefly, certainly not exhaustively, some reports of adhesives in the Digenea. We also acknowledge the roles of other anterior secretions from more conspicuous glands known primarily in miracidia and cercariae.

7.1. Miracidia

Gland cells are often conspicuous in miracidia, but are generally thought to be involved mostly with penetration of the first intermediate host, which is usually a mollusc. Once a host has been located, initial attachment is achieved using an apical papilla, a muscular organ that in some miracidia can generate suction (e.g. Wilson, 1969; LoVerde, 1975), and can be supplied with ducts from gland cells known as the 'apical glands'. Pan (1980) noted differences in the structure of the apical papilla ('terebratorium' in their terminology) between miracidia of *Schistosoma mansoni* and *Fasciola hepatica* and suggested this may have some functional significance. The function of apical gland(s) is generally considered to be histolytic to accomplish enzymatic digestion of the host's epidermis and allow penetration by miracidia (e.g. for *F. hepatica,* see Mattes, 1949; for *Neodiplostomum intermedium,* see Pearson, 1961). Descriptions of these and other miracidia refer to the presence of 'cephalic glands', 'lateral glands' or 'accessory glands' anteriorly, in addition to the apical glands (see also Wilson, 1971). It is possible that these 'cephalic glands' may secrete an adhesive to stabilize the miracidium during initial release of the contents of the apical glands in early stages of penetration. Modern labelling techniques using lectins indicate that miracidia of *S. mansoni* produce water-insoluble secretions that form fibrillar material at the site of attachment (Linder, 1986); similar observations have been made for schistosome cercariae (see below).

The ultrastructure of the apical and accessory glands of the miracidia of *F. hepatica* (see Wilson, 1971) and those of *S. mansoni* (see Pan, 1980) are similar and the miracidia of these species maintain their gland cells for several days after entering mollusc tissue (Pan, 1980). This prompted Pan (1980) to suggest that an additional function for these glands, as well as aiding entry into the mollusc, was to prepare sites inside the intermediate host for further intramolluscan development.

7.2. Cercariae

Gland cells in most cercariae are even more conspicuous than those in miracidia. These glands are thought to serve discrete functions and reflect the

variety of tasks that cercariae must complete during their short lifetime. Cercariae must escape from their intermediate host perhaps using escape glands (but there is little direct evidence that this occurs in most cercariae (Kearn, 1998) other than the schistosomes (Dorsey, 1974)). Some cercariae may need to penetrate the next intermediate host or the definitive host using penetration glands and these have been demonstrated to secrete a serine protease in Diplostomatidae and Plagiorchiidae (see Moczon, 1996b). Those cercariae that encyst possess cystogenous cells that provide material(s) to form the cyst.

Cercariae of human schistosomes have been studied most thoroughly because of their economic importance and therefore the anatomy of their gland cells is well known. Penetration of human skin is achieved by the pre-acetabular and post-acetabular gland cells, the ducts of which enter the head capsule of the cercaria. However, secretions from the post-acetabular glands that are released before penetration in *S. mansoni* may have an adhesive role during the period when the cercariae explore the surface of the skin (Robson and Erasmus, 1970). This is consistent with the discovery by Linder (1985, 1986), using lectin-labelling, that secretions from the post-acetabular glands of *S. mansoni* appeared to function as a glue. Material produced was fibrillar, comprised water-insoluble carbohydrates and was often left as two crescent-shaped spots (Linder, 1985) or 'kissing marks' (Linder, 1986).

Whitfield *et al.* (1975) briefly reviewed some examples of cercariae that use glandular secretions from the head. Maejima *et al.* (1988) used histochemical stains on the cercaria of *Gigantobilharzia sturniae* (Schistosomatidae) and revealed three pairs of what they called post-acetabular 'adhesive gland cells' that stained positively with PAS. They also identified the six duct endings which stained with silver nitrate at the anterior end. Ismail and Arif (1991) described a pair of lateral 'adhesive pockets' in their *Cercaria emirati* IV from the gastropod, *Melanoides tuberculatus,* but offered no evidence to support their claim that these structures had adhesive (in the sense of a glue) properties.

The cercariae of some flukes are reported to use an adhesive to establish contact with the surface of their host. Cercariae of *Transversotrema patialensis* swim such that their two tail arms that bear receptors and adhesive pads contact a substrate as they sink passively in the water column. Once the receptors recognize a suitable host, whether during active swimming or passive sinking, the arms rotate so that both the adhesive pads contact and anchor the cercaria to the host fish skin surface. Then by further rotation of the body, the ventral sucker can achieve firm attachment to the fish epidermis. This instant contact adhesion is achieved by membrane-bounded 'adhesive granules' that occur in the bounding plasma membrane of the pad region (Whitfield *et al.*, 1975). Activation of the pads by contact with a suitable substrate causes release of the contents of the large peripheral adhesive granules onto the pad surface to effect adhesion (Whitfield *et al.*, 1975). This system is very different from

the usual arrangement of discrete gland cells that occur elsewhere in the platyhelminths.

Adhesives are also employed by the cercariae of the bucephalids *Prosorhynchus crucibulum* and *P. squamatus*, but in these cases the two cercarial tail furcae are coated by a sticky substance (Matthews, 1973). Similarly, the base of the tail stem bears an adhesive zone in *P. crucibulum* or a trilobed arrangement in *P. squamatus*, and these surfaces also secrete adhesive substances. The furcae of each species make initial contact with their host fish's skin by adhering or entangling in projections such as scales, fins and gill rakers (Matthews, 1973). After initial adherence, the furcae contract and bring the posterior end of the cercarial body into contact with the host when the adhesive areas of the tail stem achieve secure attachment (Matthews, 1973).

Cercariae of other digeneans are known to use adhesives for a function other than attaching themselves to their next host prior to, or during, the initial stages of penetration. Some cercariae possess a gland cell or gland cells at the tip of their tail. Rees (1967) described a specialized attachment organ at the tip of the tail of cercariae of *Parorchis acanthus* (Philophthalmidae) comprising a pit into which gland ducts containing PAS-positive secretion open; these glands were thought to provide adhesive for temporary attachment to a substrate before encystation. The tail of *Cercaria queenslandae* VIII (ascribed tentatively to the Gyliauchenidae by Cannon, 1978) bears terminal invaginations into which six pairs of glands open (Cannon, 1978). When the structure is evaginated, it is reminiscent of the attached anterior adhesive areas of a monopisthocotylean monogenean (compare figure 5d in Cannon, 1978, of the 'cercarial tail cup' with our Figures 12C, D), but its significance is unknown currently. Other cercariae, such as the bivesiculid *Paucivitellosus fragilis*, have gland cells at the opposite end of the tail, but they assume importance when the cercarial head is pulled inside the caudal chamber of the tail (Pearson, 1968). Bivesiculid cercariae attach themselves to surfaces likely to be grazed by the definitive host.

Zygocercous cercariae aggregate in large numbers, connect together and move as one organism. Beuret and Pearson (1994) described this process for cercariae from *Clypeomorus batillariaeformis*, an intertidal gastropod from the Great Barrier Reef. These cercariae form large aggregates of up to 700 individuals and it was suggested that these zygocercariae first attach by the prehensile portion of their tail and that adhesion of the posterior half of the inflated part of the tail then occurs (Beuret and Pearson, 1994). These authors suggested that adhesive properties in zygocercariae may be widespread. Aggregation like this is believed to be a strategy to attract consumption by the next host in the life cycle. Perhaps the shape of the cercarial cluster mimics a free-swimming organism normally ingested by the definitive host (Combes, 1980).

The foregoing paragraphs indicate that while secretions thought to have an

adhesive function are known or suspected either within the body and/or tail of the cercariae of many families of digeneans, their adhesive properties have received relatively little attention.

7.3. Adults

Gland cells are described occasionally in association with the suckers of some mature digeneans (e.g. Halton, 1967; Halton and Dermott, 1967) and mucopolysaccharide secretions were tentatively implicated in adhesion or extracorporeal digestion by Halton and Dermott (1967). In addition to suckers, mechanical specializations for attachment in adult flukes include: a spiny, retractable proboscis, reminiscent of the proboscis of acanthocephalans, on either side of the oral sucker in *Rhopalias* spp. (Rhopaliidae) from opossums; so-called 'adhesive' or 'tribocytic' organs just posterior to the oral sucker in some strigeoids (e.g. Diplostomatidae, Strigeidae); peg-like spines on the circumoral collar of echinostomatids; the 'rhynchus' of bucephalids. The tribocytic organs are thought to secrete proteolytic enzymes for extracorporeal digestion of host mucosa (Erasmus and Öhman, 1965; Erasmus, 1970, 1972) and adhesives have not been implicated. The bucephalid rhynchus (Figure 22), however, deserves closer attention because it is sometimes referred to as an 'anterior adhesive organ' (Roberts and Janovy, 1996), it may be tentaculate (Kearn, 1998) and there are some reports of associated adhesive glands (Swarup and Jain, 1984).

Bucephalids are most distinctive because the mouth of their cercariae and adults is mid-ventral, not terminal, and it connects by a sucker considered to be the pharynx with a sac-like gut (Kearn, 1998). In most bucephalids, the anterior part of the body where the oral sucker occurs in most other digeneans can form the rhynchus (Figure 22). The rhynchus is morphologically diverse, usually muscular and can end in a disk-like structure or can bear eversible tentacles (Kearn, 1998). Figure 22 indicates that *Bucephalus sextentaculatus,* as its name implies, may possess tentacles, but these are not everted in the specimen prepared for SEM. Some bucephalids have a rhynchus that can bear rows of spines (Moravec and Sey, 1989; see also Figure 22) and there is a report of the presence of a pair of glands inside the rhynchus of *Bucephalopsis lateroporus* (see Swarup and Jain, 1984). We suspect that further study will indicate that other adult bucephalids produce adhesive secretions, but the reason why this should be so for this digenean family but not for others is obscure. The development of the rhynchus, however, indicates considerable specialization by bucephalids. The observations on bucephalid locomotion by Matthews (1973) suggest that effective attachment during movement inside the intestine of the host is of particular importance in their biology.

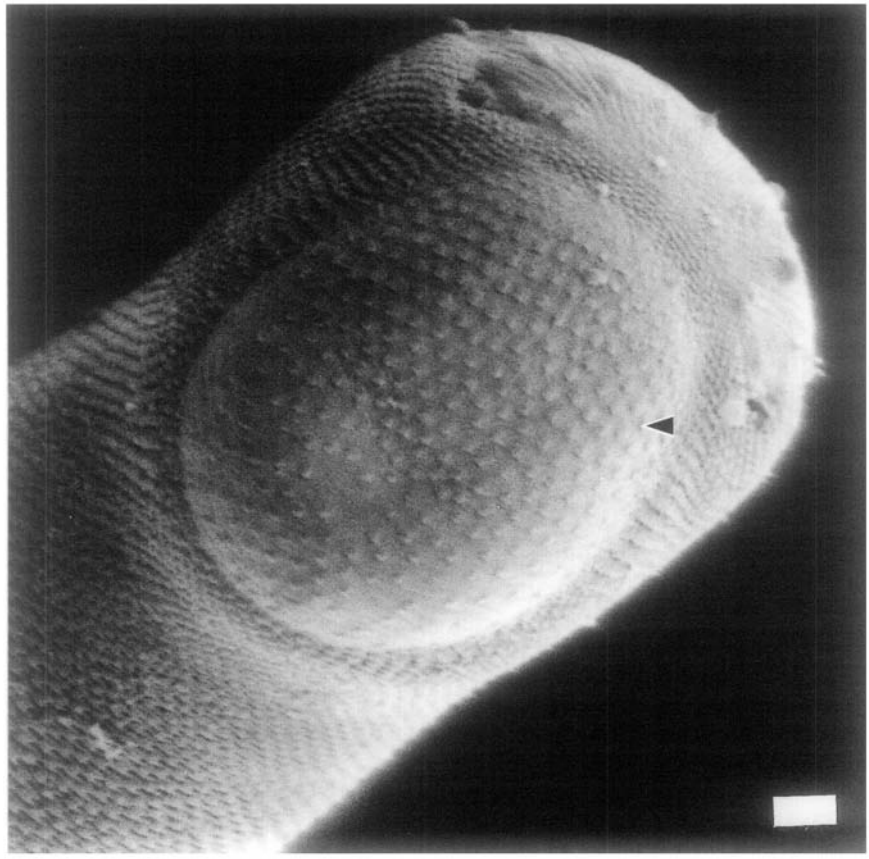

Figure 22 Scanning electron micrograph of *Bucephalus sextentaculatus* (Digenea: Bucephalidae) from the intestine of *Caranx sexfasciatus* (Carangidae) collected at Stradbroke Island, Moreton Bay, Queensland, Australia, showing the rhynchus (arrowhead), an organ that occurs anteriorly in bucephalids instead of the more familiar oral sucker found in many digeneans (e.g. Figure 3B). In this specimen, the tentacles are not everted. Scale bar = 10 μm. (B.W. Cribb and I.D. Whittington, original photograph from a specimen provided and identified tentatively by Dr T.H. Cribb.)

Attachment organs that may turn out to have adhesive components have been described in some adult digeneans of the families Gymnophallidae, Microphallidae and Paramphistomatidae. A ventral pit that may function as an accessory 'adhesive organ' was described by Seo *et al.* (1995) and Choi *et al.* (1995) in the metacercaria and adult of *Gymnophalloides seoi* (Gymnophallidae), but no mention was made of adhesives and it appears that this structure is similar to the tribocytic organ of strigeoids (see above). Well

developed accessory 'adhesive pits' that are provided with large concentrations of uninucleate gland cells are known in some microphallids (e.g. *Microphallus pygmaeus, M. piriformes* and *M. triangulatus*; see Galaktionov, 1983, 1984). The ventral surface of *Gastrodiscus aegyptiacus* (Paramphistomatidae) was reported, using SEM, to be covered completely by 'cauliflower-like' papillae which, in the opinion of Hiekal (1992), possibly have an adhesive function. Further studies of this diversity of adhesive structures in digeneans are required to determine the extent, if any, of adhesive secretions associated with these organs.

7.4. Summary

It remains a possibility that there are many more examples to be discovered where adhesives are used for attachment among digeneans, especially in stages that must actively locate the next host in the life cycle. Adhesive secretions in these cases may be useful for initial attachment to the host. However the relative speed of penetration of molluscs by miracidia or penetration of a variety of intermediate and/or definitive hosts by cercariae suggests that the role of adhesives, when present, is only brief. The basic body plan of adult digeneans with its various combinations of oral and/or ventral suckers appears to cope with the demands for firm attachment and movement in the great diversity of hosts that they infect. Adhesives, therefore, in adult digeneans appear relatively rare, but further study, especially of the bucephalid rhynchus, may be fruitful. It is no surprise that few details are known about the chemistry of digenean adhesives, although adhesives from the post-acetabular glands of *S. mansoni* cercariae are secretions of highly polymerized water-insoluble carbohydrate (Linder, 1985). So little attention has been paid to potential adhesives in the Digenea that much remains to be discovered about their presence, ultrastructure and chemistry.

8. THE ASPIDOGASTREA

The Aspidogastrea, although a minor group of the parasitic Platyhelminthes (see Rohde, 1994c) in terms of diversity, numbers of species and host range, occupies an important phylogenetic position. Aspidogastreans are considered to be primitive neodermatans and represent either the sister group to the Digenea or the sister group to all other Neodermata (see Rohde, 1994b). Most classification schemes place the Aspidogastrea as a subclass in the Trematoda. Comparatively little is known of their biology.

Fredericksen (1978) provided a detailed description of the ciliated

cotylocidium of *Cotylogaster occidentalis*. Its cephalic glands produce irregularly-shaped, slightly electron-dense bodies that are not membrane-bound and have a finely granular matrix. No histochemistry was performed and Fredericksen (1978) appeared reluctant to ascribe a function to these cells and their products, but he did draw attention to the 'adhesive characteristics' of aspidogastreans and suggested adhesion was a likely role. The cotylocidia of other aspidogastreans lack cilia and probably infect their molluscan hosts when the eggs that contain them are ingested. Rohde (1994c) and Kearn (1998) should be consulted for further details of the variety of life cycles that are known.

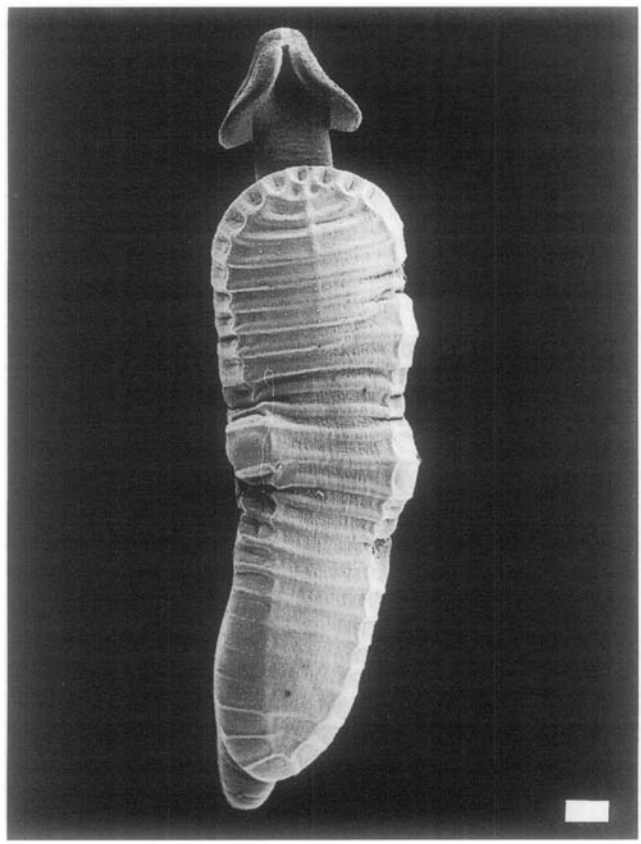

Figure 23 Scanning electron micrograph of *Lobatostoma manteri* (Aspidogastrea) from the intestine of *Trachinotus blochii* (Carangidae) from Heron Island, Queensland, Australia, showing the large and elaborate ventral attachment organ. Scale bar = 100 μm. (B.W. Cribb and I.D. Whittington, original photograph from a specimen provided by Dr T.H. Cribb.)

Adult aspidogastreans have an elaborate, ventral organ (Figure 23) usually bearing many separate alveoli responsible for mechanical attachment to the host. An abundance of sensilla on the bodies of aspidogastreans prompted Kearn (1998) to suggest that these sense organs were related to the shuffling movements of the ventral alveoli reported for these parasites. He considered that the sensilla conveyed information about the spatial arrangement of the individual suckers to the worm during locomotion. Kearn (1998) also reviewed suggestions that the aspidogastrean attachment organ may have a secondary role in feeding and cited evidence that 'marginal bodies', also known as 'marginal glands' (Fried and Haseeb, 1991; Rohde, 1994c), comprised gland reservoirs containing esterases and/or phosphatases. The ultrastructure of these gland cells in *Aspidogaster conchicola* was described by Halton and Lyness (1971) and the secretory bodies they produce are membrane-bound, spherical, electron-dense and homogeneous with a diameter of up to 1 μm. Fried and Haseeb (1991) wrote that the function of the marginal glands was equivocal. Rohde (1994c) summarized knowledge of the structure of marginal glands, which are the terminal parts of secretory ducts. In *Lobatostoma manteri*, the gland duct widens to form an ampulla which itself opens to the exterior via a narrow duct onto the surface of the worm (Rohde, 1994c). The gland cells secrete rounded, electron-dense bodies. Studies by Timofeeva (1972) using light microscopy on *A. limacoides* identified two types of unicellular gland cells in the lateral alveoli of the attachment organ and she suggested that their secretion may serve in attachment and locomotion.

9. COMPARISON OF PLATYHELMINTH ADHESIVES WITH TEMPORARY ADHESIVES IN OTHER MARINE MACROINVERTEBRATES

Temporary adhesion (Section 3.3) is known for a range of marine invertebrates other than platyhelminths, including gastrotrichs (Tyler and Rieger, 1980), polychaetes (Gelder and Tyler, 1986) and nematodes (Tyler and Melanson, 1979), but there are few examples from marine macroinvertebrates of temporary adhesives that have been characterized chemically. The composition of adhesives in echinoderms (see review by Flammang, 1996; Flammang *et al.*, 1998) and limpets (Mollusca) (see Grenon and Walker, 1978, 1980; Denny, 1983; Smith, 1991, 1992; A.M. Smith *et al.*, 1999) is better characterized than most. They differ from adhesives involved in permanent adhesion such as those reported for marine mussels (Mollusca) which use proteinaceous byssal threads and plaques (Cook, 1970; Waite and Tanzer, 1981; Waite, 1983, 1985; Waite *et al.*, 1989; Rzepecki *et al.*, 1992; Papov *et al.*, 1995). They also differ from the permanent adhesives of barnacles (Crustacea) (see Walker,

1972; Barnes and Blackstock, 1974; Walker and Youngson, 1975; Yule and Walker, 1987; Naldrett, 1993; Kamino *et al.*, 1996) and tube-dwelling worms (Jensen and Morse, 1988) which use a proteinaceous cement. Mussels make use of a quinone-tanning process involving the amino acid, 3, 4-dihydroxy-phenylalanine (DOPA), to cross-link the adhesive (Waite and Tanzer, 1981), whereas barnacles use disulphide bonds and non-covalent linkages (Naldrett, 1993; Kamino *et al.*, 1996) in a similar fashion to the protein component of limpet adhesive (A.M. Smith *et al.*, 1999).

Echinoderms use adhesives to attach their podia (= 'tube feet') temporarily to substrata during locomotion, feeding and burrowing; this area is reviewed by Flammang (1996). The adhesive system in those echinoderms that have been studied has similarities with platyhelminth adhesive systems and so is worth consideration here for comparative purposes. The chemistry and mode of action of adhesives in echinoderms are still not fully understood, but a considerable amount of useful data are available. Echinoderm podia contain a number of secretory cells in their epidermis (Flammang, 1996). These cells fall into two categories referred to as: (1) secretory or non-ciliated secretory cells, comprising one or two types, depending on the taxa (two in *Asterias rubens*, see below); (2) neurosecretory-like or ciliated secretory cells (Flammang, 1996; Flammang *et al.*, 1998). There is morphological, cytochemical and chemical evidence that supports the concept of a duo-gland system of adhesion and de-adhesion in echinoderms (Hermans, 1983), where the secretory cell(s) produces the adhesive and the neurosecretory-like cells are responsible for de-adhesion (Flammang *et al.*, 1991, 1998; Flammang, 1996).

Secretions thought to be adhesive are packaged in electron-dense, membrane-bound granules (Flammang, 1996). Studies of cells that produce these secretions show that the secretory granules are closely associated with Golgi membranes and rough endoplasmic reticulum. The ultrastructure of these granules varies from one taxon to another, with five broad categories recognized (Flammang, 1996). Secretions are released to the surface via pores or through the tips of microvillar-like cell projections. Neurosecretory-like cells (the putative de-adhesive cells) are rich in rough endoplasmic reticulum with small Golgi apparatus and produce small, electron-dense granules (Flammang, 1996). The adhesive is a fibrillar matrix which is left behind as a podial print about 5 μm thick (Flammang *et al.*, 1994; Flammang, 1996). The extruded adhesive print is insoluble (Flammang *et al.*, 1998). De-adhesion appears to occur between the surface of the podium and the adhesive material (Flammang, 1996). An overlying 'fuzzy coat' on the podia is absent after de-adhesion and is thought to be removed during the process of detachment (Flammang, 1996).

The significance of the presence of one or two different adhesive secretory granules in different echinoderm taxa is obscure. Data from histochemical studies available for the adhesive secretory granules of several species from different classes (reviewed by Flammang, 1996) show that those of most

species contain acid mucopolysaccharides, but some contain both mucopolysaccharides and protein. The variable composition is thought to reflect a requirement for adhesives with different strengths (Flammang, 1996). Interestingly, granules from the neurosecretory-like cells do not stain for acid mucopolysaccharides and do not stain with classical histochemical agents. It is considered that they may contain enzymes (Flammang, 1996).

A study of the podial prints and podia of *Asterias rubens* (Echinodermata: Asteroidea: Forcipulatida) using immunocytochemistry showed that the podial print contained material comprising the two types of putative adhesive secretory granules and the fuzzy coat from the cuticle (Flammang *et al.*, 1998). They found no labelling by antibodies raised to the podial print material on granules produced by the neurosecretory-like cell indicating that this secretion was excluded from the podial print. The podial print material is composed primarily of proteins, but carbohydrates and lipids are also present in significant amounts (Flammang *et al.*, 1998). The protein moiety consists of significant amounts of both charged (especially acidic) and uncharged polar residues as well as half-cystine. The carbohydrate moiety is likely to be acidic containing uronic acids and sulphate groups. The lipid component may come from the membranes of secretory granules (Flammang *et al.*, 1998).

Flammang *et al.* (1998) have put forward a model for adhesion and de-adhesion which is best supported by their evidence from *A. rubens*. They propose that the two secretory types released from the non-ciliated secretory cells mix physically, and possibly react chemically, to form the adhesive layer. This adhesive interacts with both the substrate and the fuzzy coat that overlies the sea star cuticle through ionic bonds. As both the protein and carbohydrate moieties contain acidic residues (and cationic gold binds heavily to the fuzzy coat of the cuticle; Flammang, 1996), these are likely to be involved in the ionic bonds (Flammang *et al.*, 1998). It is suggested that the cohesive strength could be achieved through intermolecular disulphide bonds. De-adhesion may then be brought about by the contents of the granules produced by the neurosecretory-like, ciliated secretory cells. These appear to be released just beneath the cuticle of the sea star and result in the fuzzy coat being lifted off and left on the adhesive after detachment (Flammang *et al.*, 1994, 1998; Flammang, 1996). This evidence again points to the action of an enzyme as the de-adhesive agent (Flammang, 1996; Flammang *et al.*, 1998).

As Flammang *et al.* (1998) stated, the closest system to the temporary adhesion of echinoderms which has been characterized chemically is seen in the pedal adhesive secretions of limpets, e.g. *Patella vulgata* (Mollusca: Gastropoda), which can use their secretion as a viscous fluid during locomotion or as an adhesive when sessile (Grenon and Walker, 1980; Denny, 1983; Smith, 1991, 1992; A.M. Smith *et al.*, 1999). Limpets produce an adhesive which is also a protein–carbohydrate complex. The carbohydrate is a sulphated mucopolysaccharide and links to the protein moiety by electrovalent

bonds (Grenon and Walker, 1980; Naldrett, 1993; Kamino *et al.*, 1996; A.M. Smith *et al.*, 1999). Data reviewed here suggest that this chemistry is ubiquitous in temporary, mucus-like adhesives. In contrast, the permanent adhesive of the mussel *Mytilus edulus* (Mollusca: Bivalvia) contains proteins predominantly and has no carbohydrates (Cook, 1970), whereas that of the barnacle, *Balanus crenatus* (Crustacea: Cirrepedia), contains a little carbohydrate but is again predominantly proteinaceous (Walker, 1972). Barnacle cyprid larvae also produce an antennular adhesive secretion which is composed entirely of protein (Walker and Yule, 1984). Interestingly, the limpet, *Lottia limatula* (Mollusca: Gastropoda) modifies the composition of its secretion to form either the gliding mucus substance or the adhesive: the protein fraction changes and twice the amount of protein and carbohydrate are present in the adhesive secretion (A.M. Smith *et al.*, 1999). The possibility of either modification of a single secretion, as determined in limpets, or mixing of a variety of secretions, as appears possible in echinoderms, presents a flexible variety of secretions used for temporary adhesion. The next step in characterization will undoubtedly concentrate on how modification of protein and carbohydrate fractions lead to changes in adhesive properties among bioadhesives.

There are a number of similarities between the echinoderm adhesive system and that of platyhelminths. Perhaps the most comprehensively studied flatworm group and that with the most convincing claim to a system for firm but short-term adhesion, besides the Monogenea, are the turbellarians. In many Turbellaria, a duo-gland system named by Tyler (1976) and discussed by Hermans (1983) is proposed and comprises a viscid (adhesive) gland and a releasing gland (Section 4.4). It must be remembered, however, that the proposed explanation for the mechanism of the duo-gland system of turbellarians is based on circumstantial evidence (Section 4.4, p. 123–124) and that functional studies are difficult technically because of the small size of the organs and organisms. The mode of operation of the duo-gland system in Turbellaria, therefore, remains speculative (Tyler, 1976, 1988; Section 4.10, p. 139). Nevertheless, the structural arrangement of the putative duo-gland system in Turbellaria is similar to echinoderm systems where one adhesive secretion is present, but not when more are present. In turbellarians, there can be no mixing of different secretions to produce an adhesive (but see Ehlers' (1989) on the kalyptorhynch, *Schizochilus caecus*; Section 4.5, p. 126). In some cases, the histochemistry of the secretions produced by turbellarians and echinoderms is also similar. Like the limpet system (A.M. Smith *et al.*, 1999), the viscid secretion of turbellarians contains protein and polysaccharide (proteoglycan?) and demonstrates sulphydryl groups (Tyler, 1988). As determined for the echinoderm system (this Section, see p. 196), the putative releasing gland secretion in turbellarians does not react in histochemical tests (Tyler, 1988) and may therefore correspond with the secretory granules from echinoderm neurosecretory-like cells. This may suggest, perhaps, that this second secretion in

turbellarians may comprise an enzyme unlike a previous model proposed to explain de-adhesion in the duo-gland system (Hermans, 1983): release of polyanionic substances (such as acid mucopolysaccharides) that competed for active sites on the adhesive molecules (polycationic substances such as basic residues of adhesive proteins) were thought to out-compete their bonds to the anionic sites of the substrate.

For the Monogenea, there has been no chemical characterization of putative adhesive secretions beyond histochemistry, except for work in progress by Hamwood, Cribb, Halliday, Kearn and Whittington (unpublished data), but some useful comparisons with echinoderms can be drawn. Within the Monogenea, histochemical data tell us that some monopisthocotyleans use a posterior adhesive secretion which is a tyrosine-rich lipoprotein (Rand *et al.*, 1986), but it is the anterior adhesive system of monogeneans which is most reminiscent of the echinoderm adhesive system. Similarities evident are: the presence of multiple secretions released from ducts which may mix to form the adhesive and/or a separate de-adhesive secretion; the ultrastructure of putatively adhesive spherical secretions may differ across monogenean taxa (Kritsky, 1978; El-Naggar and Kearn, 1980, 1983; Rees and Kearn, 1984; Yuan and Long, 1996; Yuan and Lang, 1997; Cribb *et al.*, 1998; Whittington and Cribb, 1998a, 1999); adhesive prints remain after adhesion to glass (Kearn and Evans-Gowing, 1998); adhesive area tegument remains free of adhesive after de-adhesion from a substrate (Kearn and Evans-Gowing,1998), possibly pointing to a role for tegumental secretions such as enzymes, in de-adhesion. Conversely, there are some distinct differences between the anterior adhesive systems of monogeneans and the system of adhesion in echinoderms. A major and perhaps significant difference is that echinoderms generally use their adhesive system for temporary attachment to mostly inert substrates during their activities of movement, feeding and burrowing (Flammang, 1996). Monogeneans, however, use their anterior adhesives for temporary attachment to living surfaces, adhesion we have termed tissue adhesion (Section 3.4). Structural and chemical differences between adhesive systems in echinoderms and monogeneans include: rod-shaped secretions (absent from echinoderms) appear to comprise the bulk of the adhesive, rather than spherical secretory bodies, in almost all monogenean species for which ultrastructure (Table 1) has been investigated (El-Naggar and Kearn, 1983; Rees and Kearn, 1984; Cribb *et al.*, 1997, 1998; Whittington and Cribb, 1998a, 1999); rod-like bodies are remarkably similar in morphology between taxa (Kritsky, 1978; El-Naggar and Kearn, 1980, 1983; Rees and Kearn, 1984; Cribb *et al.*, 1997, 1998; Whittington and Cribb, 1998a, 1999), possibly pointing to a conserved chemistry for this component within the Monopisthocotylea; no demonstration of a carbohydrate component in extruded adhesive, only amino acids; the tegument of the adhesive region is morphologically specialized differing from the general body tegument and short microvilli are always present (Kritsky, 1978;

El-Naggar and Kearn, 1980, 1983; Rees and Kearn, 1984; Yuan and Long, 1996; Yuan and Lang, 1997; Whittington and Cribb, 1999, and Figure 17). If the specialized anterior adhesive area tegument in monogeneans does play a role in de-adhesion (Kearn and Evans-Gowing, 1998), then these observations suggest that the secretion involved is produced by the whole of the specialized tegument rather than from gland cells which penetrate through it or end blindly beneath its outer layer as is the case for echinoderms.

Those secretions that may have an adhesive function in cestodes (Sections 6.1 and 6.2) appear to be glycoproteins (Hayunga, 1979a: Kuperman and Davydov, 1982b; McCullough and Fairweather, 1989; Brockerhoff and Jones, 1995). However mucopolysaccharides in penetration glands (Section 6.1) may also have an adhesive role (Kashin, 1986). In the Digenea, adults secrete mucopolysaccharides (Section 7.3) which have been implicated tentatively in adhesion (Halton and Dermott, 1967). After attachment, cercariae of *Schistosoma mansoni* show a crescent-shaped residue (Section 7.2) which is a fibrillar, water-insoluble carbohydrate (Linder, 1985, 1986). At this stage, information for cestodes and digeneans is too limited to enable useful comparisons with other known adhesive systems.

Much research remains to be done in the area of chemical and functional characterization of platyhelminth adhesives to achieve a similar level of understanding for comparison with the better characterized systems reviewed above. From available data, some chemical similarity between echinoderm, limpet and platyhelminth adhesive systems exists. A.M. Smith *et al.* (1999) highlight the major hindrance to progress in the area of temporary adhesives: a lack of direct evidence that identifies which components are responsible for adhesion. Such information appears to be attainable by immunocytochemical studies such as those of Flammang *et al.* (1998) on echinoderms. Another approach is to apply techniques of rapid fixation during carefully timed experimental observations before, during and after temporary adhesion, followed by ultrastructural investigations. These procedures have yielded significant preliminary information on anterior attachment and detachment by adhesives in the capsalid monogenean, *Entobdella soleae* (see Kearn and Evans-Gowing, 1998).

10. CONCLUDING COMMENTS

Our review is the first attempt to draw together information about use of adhesion, in the sense of attachment by chemical means via a thin layer of adhesive material, by symbiotic or parasitic platyhelminths, or indeed by any parasite group, to a host organism. Adhesion by adhesins (i.e. surface molecules known from, and characterized in, some bacteria and protists that invade host cells) is

an active, but currently separate, research field (e.g. Alderete, 1999; Kennett *et al.*, 1999). Future studies may indicate whether there are similarities in the molecular interactions between these invaders and their host cells and the bioadhesives secreted for attachment to their host's surfaces by, for example, the flatworms reviewed here.

The ubiquity of adhesion in nature was referred to in Section 2. The burgeoning discipline of bioadhesion is testimony to the innate curiosity, interest and importance placed on understanding how adhesion in nature occurs. Other outcomes from bioadhesion studies not only include attempts to replicate in the laboratory or in industry methods of producing such tenacious natural adhesives which are known for their toughness (B.L. Smith *et al.*, 1999), but also embrace development of methods to *prevent* some organisms from adhering (i.e. production of antifoulants). Marine invertebrates are a particularly rich source of natural adhesives and, as Flammang (1996) phrased it so clearly, 'adhesion is a way of life in the sea'. Many marine invertebrates with a benthic or interstitial lifestyle have evolved various organs, organelles and/or secretions for adhesion to different substrates. Generally, however, these surfaces are non-living (i.e. abiotic). We have emphasized that some parasitic organisms, many from marine vertebrate hosts, have developed mechanisms by which they can adhere to a living (i.e. biotic) surface. We believe that adhesion to living surfaces (= tissue adhesion; Section 3.4) represents an exciting extension to the arena of bioadhesion.

Tissue adhesion has important implications for the biology of organisms, especially parasites such as monogeneans and animals like some barnacles, that have evolved effective systems to adhere to epithelia. Substrates such as vertebrate epithelia are moist, usually slimy and covered by a mucous layer and are equally as inhospitable as intertidal rocky zones and ship hulls, surfaces commonly occupied by invertebrates such as mussels and barnacles and subject to strong shear forces from tidal action. Epidermis could, perhaps, be considered *more* hostile than abiotic substrates in the ocean because they may contain elements of the immune system. We hope the concept of tissue adhesion will provide a catalyst for further study in parasitology. These studies should include: the potential importance of adhesives in host- and site-specificity; the role of adhesives in provoking an immune response from a host; how attachment by adhesives may contribute to the co-evolutionary arms race between parasites and hosts (Whittington *et al.*, 2000a). Host- and site-selection by parasites based on adhesive–host substrate recognition may resemble the phenomenon of 'habitat selection' (i.e. choice of favourable versus unfavourable, but inert, surfaces) by pelagic larvae of free-living, benthic marine invertebrates (Hadfield, 1998; Whittington *et al.*, 2000a).

Permanent adhesion to abiotic substrates by marine organisms such as mussels and barnacles is achieved principally by proteinaceous secretions that contain little or no carbohydrate (Section 9). The chemistry of temporary

adhesives for attachment to abiotic substrates is poorly characterized (Flammang, 1996), but studies on limpets and starfish indicate that complexes of proteins and carbohydrates are common (Section 9). A.M. Smith *et al.* (1999) discovered that the limpet, *Lottia limatula* (Mollusca: Gastropoda), is able to change the amount of protein and carbohydrate in its single adhesive secretion and this difference in proportion changes its properties to allow either gliding or adhesion. Similarly for starfish, the ability to vary the ratio of acid mucopolysaccharides to protein across taxa (Flammang, 1996) may alter the strength of the adhesive. In comparison with these marine macroinvertebrates, the chemistry of adhesives in platyhelminths is virtually unknown, largely because of their small size. Indications on present evidence suggest strongly, however, that proteins and, in some cases carbohydrates, are also important in flatworm adhesives. The duo-gland system of turbellarians has proteinaceous and glycan components and is perhaps a glycoprotein (Tyler, 1988). In monogeneans (Section 5), anterior adhesive secretions are proteins (Hamwood, Cribb, Halliday, Kearn and Whittington, unpublished data) and posterior cements are lipoprotein (Rand *et al.*, 1986). Secretions implicated in adhesion among cestodes (Section 6) are mucopolysaccharides in an oncosphere (Kashin, 1986), and glycoproteins in a metacestode (Brockerhoff and Jones, 1995) and in adults (McCullough and Fairweather, 1989; Stoitsova *et al.*, 1997). The posterior adhesive from the rosette organ in a gyrocotylidean has been characterized as a mucoprotein (Lyons, 1969).

For turbellarians (Section 4) in which there is usually more than one secretory type in their adhesive systems, there is no clear information about possible interactions or chemical differences between different components despite ultrastructural studies of many taxa. In the monogeneans (Section 5), anterior adhesive secretions of only 15 species from five families (Table 1) have been studied in detail. There is usually more than one secretory type released anteriorly by monogeneans and several hypotheses have been proposed about their possible interactions (Section 5.2.2(e), p. 157). Most information is available for the capsalid monogenean, *Entobdella soleae*, from work by Kearn and Evans-Gowing (1998). They presented evidence to suggest that two secretory types interact to produce the adhesive and some evidence to indicate that the specialized tegument of the anterior adhesive areas may be the agent responsible for detachment (Kearn and Evans-Gowing, 1998). There is clearly a need to investigate anterior adhesion in more monogenean species from a range of parasite families, and from a diversity of host taxa, to determine more about interactions between different secretions and how detachment mechanisms operate. Future studies to contribute information to fill some of these gaps in our knowledge about platyhelminth adhesion, especially the tissue adhesion displayed by monogeneans, provide significant but exciting challenges in parasitology and in the sphere of bioadhesion.

Despite the apparent similarities in the chemical composition of adhesives

highlighted above among many marine invertebrates from different taxa, Tyler (1988) asserted that a variety of polymers could act as natural adhesives. By focusing on the so-called duo-gland adhesive system reported from various marine invertebrates (e.g. Tyler, 1976; Hermans, 1983), Tyler (1988) considered that a functional comparison, including secretion chemistry, showed that variety was encountered. Tyler (1988) proposed that this variation demonstrated that there is no universal mechanism common to the adhesive systems in these different marine organisms and that any similarities between major invertebrate groups are due to convergence. Even within the so-called duo-gland organs of turbellarians, gastrotrichs and nematodes, functional differences (specifically different polymers) demonstrate convergent morphological similarity (Tyler, 1988). Determination of which secretions function as adhesives and which may serve other purposes such as detachment ('release' or 'de-adhesion' in other terminology) were cited by Tyler (1988) as difficulties to overcome. Many of these quandaries remain for future investigations on temporary adhesion by starfish and turbellarians and for tissue adhesion by monogeneans.

Structural similarities across the variety of glandular adhesive systems reviewed here relate to the activity of secretory cells across the eukaryotes. Common cellular components and structural architecture include: unicellular gland cells containing Golgi complexes and rough endoplasmic reticulum; microtubules around some secretory bodies during their formation; microtubules that line the neck or duct endings; short microvilli on the surface of the organism close to where ducts open. Evidence for the importance of microtubules as cellular 'motors' for organelle movement and membrane traffic is increasing (Goodson *et al.*, 1997). This supports earlier suggestions that microtubules may play a role in transport of vesicles containing secretory material from Golgi apparatus during formation of turbellarian rhabdites (Lentz, 1967) and rod-shaped bodies in monogeneans (El-Naggar and Kearn, 1980). El-Naggar and Kearn (1980) discussed other possible roles for microtubules. For those surrounding forming secretory bodies, it was suggested the microtubules may orientate the rods within the lumen of the gland ducts to form parallel bundles, whereas for those lining duct terminations, it was suggested that if microtubules are contractile, they may assist secretion of bodies to the exterior (El-Naggar and Kearn, 1980). Most of these suggestions appear likely and probably explain the apparent widespread presence of microtubules in cells with secretory activity across a diversity of taxa.

The morphological similarity in adhesive systems between different phyla is very likely due to convergence: in the marine environment, for example, attachment to a variety of substrates is probably an adaptive feature (Tyler, 1988). Our review, however, has focused on adhesives in a single phylum, the Platyhelminthes, and there have been several discussions about whether there is homology between the adhesive glands of different flatworm groups, e.g.

between turbellarians and monogeneans (El-Naggar and Kearn, 1983; Rees, 1986; Kearn and Evans-Gowing, 1998; Cribb *et al.*, 1998) and between all flatworm groups (Ehlers, 1985; Rohde, 1990; Xylander, 1990; Jondelius, 1992; Littlewood *et al.*, 1999). There is general agreement that turbellarian Rhabditophora are characterized by lamellar rhabdites (Rohde, 1990; Rieger *et al.*, 1991; Littlewood *et al.*, 1999) and that turbellarian Rhabditophora (see Rohde, 1990; Littlewood *et al.*, 1999) and Macrostomida (see Littlewood *et al.*, 1999) have duo-gland adhesive organs consisting of one anchor cell and two types of gland cells. Rohde (1986) was of the opinion that further studies were required to assess homology or otherwise among anterior gland cells in the parasitic platyhelminths.

Ehlers (1985) regarded the anterior adhesive systems of monogeneans and the adhesive systems of turbellarians as fundamentally and structurally different and concluded that the duo-gland system of rhabditophoran turbellarians and the anterior adhesive systems of monopisthocotylean monogeneans have arisen convergently and are, therefore, analogous, not homologous. For further discussion, see Jondelius (1992) and Kearn and Evans-Gowing (1998). We consider that caution and further study is required before Ehlers' conclusion can be stated with any certainty. To argue against Ehlers' (1985) hypothesis of convergence, the duo-gland system of turbellarians and the anterior adhesive areas of most monopisthocotylean monogeneans have separate secretions emerging via ducts that pass through a surface cell or syncytial layer of cytoplasm provided with microvilli. After further study, it is possible that each of these systems may be shown to be the same functionally for it must be remembered that Tyler's (1976, 1988) explanations for the mode of action of the duo-gland system in turbellarians is based on speculation (Sections 3.5, 4.4, 4.10 and 9). Furthermore, some differences between the adhesive systems of these taxa may be expected should monogeneans have inherited their adhesive systems from turbellarian ancestors. In our opinion, homology or otherwise of the duo-gland system of turbellarians and the anterior adhesive areas of monopisthocotylean monogeneans remains unresolved.

It is intriguing that a major secretory product of the Turbellaria, the rhabdite (Section 4.3), known to have adhesive properties among temnocephalans (Section 4.6), bears a close resemblance in shape, if not in structure (compare Sections 4.3, 5.2 and Table 1), to rod-shaped bodies secreted by one type of anterior adhesive gland in monopisthocotylean monogeneans. While further study of the ultrastructure of monogenean rods is likely to be fruitful, rhabditophoran rhabdites appear to be more complex than monogenean rods. Llewellyn (1965) proposed that ancestral monogeneans arose from free-living rhabdocoel-like ancestors and, therefore, resemblance between the adhesive secretions and systems of monogeneans and turbellarians may reflect common ancestry. For further discussion on this, see El-Naggar and Kearn (1983). However, it remains to be determined whether the shape of these 'rhabdiform'

bodies reflects a phylogenetic link (see above) or whether it has some functional significance. Further studies on possible interactions between different secretory types may clarify this.

The continued success of a diversity of flatworms owes much to the persistent development and refinement of their attachment by adhesion, which has assumed critical importance in their biology, whether they live in the interstitial environment, or subsist as symbionts or parasites. The range of adhesive systems in platyhelminths extends beyond the duo-gland system of turbellarians (Section 4.4) and the anterior adhesive areas of monopisthocotylean monogeneans (Section 5.2). It includes the 'frontal organ' and 'frontal glands' of free-living turbellarians (Section 4.5), various glands described from symbiotic turbellarians (e.g. anterior glands in Pterastericolidae, possibly frontal glands in graffillids and other secretions, including rhabdites, from temnocephalan tentacles; Section 4.6), gland cells from the posterior end of monopisthocotylean monogeneans (Section 5.3) and putative adhesives secreted by some cestode stages (Section 6). Many of these systems may have evolved independently, especially some of the diversity of adhesive systems reported among turbellarians (Section 4). Haptor adhesive glands in some monogeneans have probably evolved more than twice (in microbothriids, anoplodiscids and in at least two genera of dactylogyrids; Section 5.3).

It is surprising that the diversity of characters known from the anterior ends of monogeneans have not been used in phylogenetic assessments based on morphology (e.g. Boeger and Kritsky, 1993, 1997, in press) and we will redress this situation in the future. The diverse morphology of the anterior adhesive areas and the number, ultrastructure and chemistry of the different secretory types seem likely to be especially informative.

Xylander (1990) considered the structure and function of the anterior glands of the lycophore larva of *Gyrocotyle urna* (Gyrocotylidea; Section 6.3). On comparing these glands with those of the larvae of other parasitic platyhelminths (the Neodermata of Ehlers, 1985), Xylander (1990) concluded that a clear correspondence between glandular systems of different types of larvae of the Neodermata was not possible. Ten years after this statement by Xylander (1990), we have reached the same conclusion following the present detailed review based on evidence from larvae and adults. However some uncertainties in our knowledge have been addressed. Xylander (1990) wrote that larval glands of all Neodermata degenerate after infection of the host and that glands in many postlarvae arise *de novo* and are not related to glands observed in larvae. Chisholm and Whittington (1996b) have since proposed the possible homology of gland cells in the oncomiracidium and adult of *Heterocotyle capricornensis* (Monogenea: Monopisthocotylea: Monocotylidae). Other similar gland homology is likely to exist more broadly across the Neodermata.

Efficient methods of temporary adhesion in turbellarians and monogeneans

probably reflect the importance of mobility to these flatworms that have relatively simple life cycles and occur mostly on external surfaces, whether inert particles and substrates or living epithelia. For cestodes and digeneans, however, the complexity of their indirect life cycles places considerable and different demands on all stages. Whether an active infective larva, a larval stage requiring passive consumption by an intermediate host or a juvenile (e.g. a metacestode or a metacercaria) inside an intermediate host waiting to be consumed, there is often less, or no, requirement for anterior adhesive glands. Instead, the emphasis for infective stages of the endoparasitic flatworms is primarily for penetration into a host or penetration through various tissues from the gut when eaten by an intermediate host. Section 6 on cestodes and Section 7 on digeneans review the diversity of gland cells at the anterior end of larval, juvenile and adult stages, but accounts of unequivocal adhesive secretions are relatively rare. It is possible, however, that use of adhesives in cestodes and flukes may have been overlooked. A conspicuous array of anterior glands are present whose primary purpose appears to be to secrete histolytic secretions for penetration into, or escape from, hosts, but whether evolution of these glands for this range of tasks is independent or not is unknown, and more studies are required.

In adult cestodes and digeneans, there is the general perception that these endoparasitic flatworms have considerably less need for mobility. Nevertheless, tapeworms are known to migrate along the gut (Kearn, 1998) and digeneans can move by looping using the oral and ventral sucker (e.g. Sukhdeo *et al.*, 1988). Adhesive secretions from the scolex of some adult eucestodes is reported and it seems likely that this phenomenon has arisen independently in different orders, although further research is necessary. It is possible that some digeneans such as the bucephalids have an increased reliance on adhesives, but this also requires confirmation. Even the efficient and large ventral attachment organ of aspidogastreans (Figure 23) is reported to be supplemented by secretions proposed to have an adhesive function (Timofeeva, 1972). Clearly, there is a need for a critical assessment after further investigation of the presence, extent, characteristics and characterization of secretions, using ultrastructural and chemical methods, among the endoparasitic platyhelminths.

Studies will continue to examine adhesion in the more easily accessible and readily observable ectoparasitic monogeneans. It will be of interest to compare the chemistry of the kinds of adhesives used by different platyhelminths for tissue adhesion to external epidermal surfaces (e.g. fish skin) by monogeneans with the less common (?) tissue adhesion by tapeworms and flukes for attachment to the lining of the vertebrate intestine and its outgrowths. We predict that convergence in the chemistry of their adhesives is most likely because these different but related parasites face similar problems of attachment to host tissue. One difficulty that investigators will face in studies of the chemistry of flatworm adhesives is obtaining sufficient quantities of material on which to work.

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The Use of Ultrasound in Schistosomiasis

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ABSTRACT

Ultrasound was introduced in the 1970s as a method to detect schistosomal pathology both at hospital and field level. It has since been established as a safe, rapid, non-invasive and relatively inexpensive technique for assessing schistosomiasis-related lesions in individual patients and in community surveys. It can be used to validate laboratory tests to measure morbidity and provides an opportunity to visualize the evolution of pathological lesions after treatment. The interpretation of ultrasound imaging depends on the experience of the investigators and it may not be the ideal tool to detect early lesions of the affected organs. This paper reviews and critically discusses the present knowledge of morbidity due to the different types of schistosomiasis as it can be observed using ultrasound, with special reference to its use as a diagnostic and monitoring tool in field surveys. It analyses the practical use, benefits and drawbacks of ultrasound investigations to assess pathological lesions due to schistosomiasis in relation to other diagnostic tools. The role of ultrasound investigations among other monitoring approaches in control programmes is discussed in the context of rational control strategies.

1. INTRODUCTION

1.1. Epidemiology of Schistosomiasis

It is currently estimated that 200 million people are infected by schistosomiasis, one of the world's most prevalent parasitic diseases. Of these, 120 million are symptomatic and 20 million suffer from severe disease (World Health Organization, 1999). Data are usually reported from national control programmes or they are extrapolated from figures of the World Health Organization (WHO) schistosomiasis atlas (Doumenge *et al.*, 1987) and applied to 1995 population estimates. The global burden of schistosomiasis morbidity results in 156 000 years of life lost and accounts for 1.5 million disability adjusted life years (DALY; years lived with disability). It ranks 6th among parasitic diseases in both categories (Murray and Lopez, 1996). The fatality rate of intestinal schistosomiasis due to *Schistosoma mansoni* is estimated to be as high as 1 in 1000 infected people in Sudan (Kheir *et al.*, 1999).

Schistosomiasis is endemic in 76 countries and territories. An estimated 85% of all cases, and virtually all of the most severe, are concentrated in African countries. The most affected country in the Americas is Brazil, with 3 million infected people; in Asia, it is China, with nearly 1 million infected, and in the Middle East, Yemen. Population growth and movement in endemic

areas, and ecological changes resulting from increasing use of water for irrigation and electricity generation, have contributed to the spread of infection. A new initiative to complete the atlas of schistosomiasis epidemiology using the computer technology of geographical information systems (GIS) is currently under way (WHO, 1999).

1.2. Status of Control

Large-scale control programmes are commonly based on chemotherapy, usually with praziquantel, and supplemented by environmental measures aiming at transmission reduction. Interventions to control morbidity at individual and at community levels are the key control operations. Target groups include school-aged children, fisherpeople and irrigation workers, and also whole communities with high prevalence rates. The capacity to diagnose and to treat, and the availability of affordable, high quality drugs in sufficient amounts as part of essential drug packages at all levels of the health system, are pivotal for the success of such programmes. Transmission control measures require a multisectoral approach including water, sanitation, and snail control. These can result in a reduction of the need for chemotherapy, as shown in control programmes in Zimbabwe (WHO, 1999).

Many attempts have been made to develop a vaccine against schistosomiasis, but a product for large-scale use that shows good cost-effectiveness is not yet available and is unlikely to become available in the next decade (Bergquist, 1998; Capron, 1998). The importance of health education, using a well tailored IEC (Information–Education–Communication approach) is widely accepted, and can be implemented through the school system, but its feasibility appears to be limited (El Katsha and Watts, 1998).

Treatment at community level results in a drop in prevalence of schistosomiasis. Since prevalence is generally higher in school-aged children, control programmes often involve both health and educational sectors. If transmission is not reduced concurrently, however, a gradual return to pre-treatment prevalence occurs. Nevertheless, repeated treatments result in short- and long-term reduction of morbidity in countries with high endemicity even without important changes in prevalence. A number of countries have successfully introduced and sustained national control programmes, achieving impressive results by approaching schistosomiasis elimination or even reaching this goal: these include Puerto Rico, Morocco, Tunisia and Saudi Arabia. Other countries with a heavy burden of schistosomiasis, such as Brazil, Egypt, the Philippines and China, have substantially reduced the burden of disease, especially its severe consequences. The current strategy in schistosomiasis control in most African countries is to concentrate on reducing morbidity, rather than attempting to eradicate the disease by transmission reduction.

Schistosomiasis control is now being tackled in a broader context, integrating the epidemiology and control of both geohelminth and *Schistosoma* infections. Although these two groups of infections may be independently distributed within countries where they are endemic (Brooker *et al.*, 1999), they both affect school-aged children, which simplifies targeting within communities. If morbidity control is to be achieved with chemotherapy, the amount of morbidity among populations due to geohelminth infections and to each *Schistosoma* species, and its distribution, must be known in order to assess the severity of the diseases. Once treatment has been administered, the pattern of regression of lesions must be followed to determine the most appropriate cycles of treatment and re-treatment.

1.3. Key Issues of Morbidity and its Assessment

The considerable knowledge that exists about the epidemiology and pathology of human schistosomiasis contrasts with the relatively limited information about the development and dynamics of the pathological changes involved, including the predictive potential of specific lesions for severe disease.

Bladder wall lesions and periportal thickening in the liver, as detected by ultrasound among individuals living in endemic areas, are pathognomonic for schistosomiasis morbidity (Tables 1 and 3). The importance of genital schistosomiasis is not yet fully assessed.

Indirect and direct methods to detect infection and pathological lesions are available to assess schistosomiasis morbidity. Indirect methods such as egg counts and other laboratory tests measure the prevalence and intensity of infection (Vennervald *et al.*, 1998). Among the latter, serological tests have the advantage of standardization compared with parasitological techniques in which samples are manually prepared. Since intensity of infection is a key determinant of morbidity, the development of markers of morbidity rather than surrogate indicators of intensity of infection are necessary. Other indirect approaches such as questionnaires reflect the amount of perceived level of disease. Direct visualizing tools, including ultrasound and other radiological techniques, document the anatomical lesions of the disease in different organ systems (see Section 3, p. 234).

The prevalence and severity of pathological abnormalities of the urinary tract are proportional to the frequency and to the number of excreted eggs in urine for *S. haematobium* infections. In *S. mansoni* and *S. japonicum* infections, the association between eggs detected in stool samples and pathological lesions is less marked. Peak morbidity is observed among children aged 6 to 15 years and in adolescence or adulthood for the parasites causing urinary tract and intestinal schistosomiasis, respectively.

Egg counts in urine and stools are the classical way to diagnose

Table 1 Development of *S. haematobium*-related pathology and its reflection in trans-abdominal ultrasound imaging in endemic areas.

Pathology	Pathognomonic lesions in endemic areas[a]	Ultrasound correlate of pathology	Detectable by ultrasonography (sensitivity)[a]
Bladder			
Nodule, 'sandy patches'	–	Irregularity of inner surface of wall	(+)
Granuloma, ulcer	–	Localized wall thickening or 'defect' on inner surface of wall	++
Pseudopolyp, mass	+	Outgrowth of wall	+++
Calcification	++	Double line of bladder wall, bright spots with conical shadows	+
Calculi	(+)	Echodense masses with conical shadows	+++
Ureter			
Nodule, 'sandy patches'	–	Not visible	–
Granuloma	–	Visible, if ureter function impaired	(+)
Obstruction	(+)	Dilatation visible	+
Kidney			
Mild congestion	–	Pyelon distended	+
Moderate congestion	–	Pyelon and calices distended, parenchyma narrowed	++
Severe congestion	–	Pyelon and calices grossly distended, thickness of parenchyma minimal or absent	+++
Upper genital organs in women	?	Enlarged uterus and ovaries, uterine, tubal and ovarial hyperechogenicities, hydrosalpinx, cervical calcification	(+)[b]
Genital organs in men	?	Dilatation and calcification of seminal vesicles, scrotal masses	(+)

[a] –, Not pathognomonic, not detectable by ultrasonography; (+), limited pathognomonic specificity or detectability by ultrasonography; + to +++, increasing degrees of good pathognomonic specificity and detectability by ultrasonography.
[b] Transrectal and scrotal ultrasonography.

schistosomal infection. Multiple examinations (three to five urine filtrations for *S. haematobium*; 3 pairs of stool preparations for *S. mansoni* and *S. japonicum*) are necessary to assess adequately the infection status of an individual. Microhaematuria, and to a limited extent proteinuria, as determined by chemical reagent strips have gained acceptance for detection of *S. haematobium* because they are less dependent on diurnal and day-to-day variations than are egg counts. Microhaematuria was also confirmed to be a useful tool for surveillance during follow-up of a control programme of *S. haematobium* with repeated chemotherapy on Pemba Island (Lwambo *et al.*, 1997).

Promising novel measurements of worm burden (*S. mansoni* and *S. haematobium*), and hence of infection intensity, are two circulating adult worm antigens, CAA (circulating anodic antigen), and CCA (circulating cathodic antigen), which are measured in serum and urine samples. Such tests have contributed to a better understanding of infection dynamics, as demonstrated by the following example: mean egg counts of *S. haematobium* fell to very low levels one month after treatment in a cohort of children in Cameroon (Kremsner *et al.*, 1994). At 12 months, egg counts rose to pretreatment levels, indicating reinfection. In contrast, antigen levels fell only to a plateau one month after treatment, and had risen to pretreatment levels by 12 months. These findings suggest that the effect of treatment is temporary, suppressing egg output rather than killing the worms.

Two other promising markers for *S. haematobium* infections are assays for a urinary soluble egg antigen (SEA) (Kahama *et al.*, 1998) and the adaptation of a urine assay for eosinophilic cationic protein (ECP, established in the diagnosis and monitoring of bronchial asthma), reflecting the eosinophilic response against eggs in the lower urinary tract (Reimert *et al.*, 2000). Both markers were associated with egg counts and with pathological lesions detected by ultrasound among infected schoolchildren in Kenya before and after treatment. Levels of SEA and egg output showed similar correlations with ultrasound detectable pathology (Kahama *et al.*, 1999). Furthermore, the SEA and ECP followed the regression and reappearance of visualized morbidity during the 18–24 months follow-up period after treatment in Tanzania (Hatz *et al.*, 1998; C.F.R. Hatz, unpublished data) more closely than egg counts and at least as well as microhaematuria measurements. In addition, minor bladder pathology at 12 months after treatment was mirrored by positive, though reduced, levels of ECP in the urine, reflecting the mild form of morbidity detected in ultrasound examinations.

Interleukins (IL-6, IL-8) measured in the urine may be additional markers of the dynamics of *S. haematobium* infection before and after treatment, disappearing within 24 hours after treatment.

To date, there is more information about associations between indirect morbidity markers and visualized pathological lesions for *S. haematobium* infections than for *S. mansoni* and *S. japonicum* infections. This is partly due

to the fact that early lesions due to intestinal schistosomiasis are less clear-cut and harder to evaluate in a standardized way.

Self-administered or guided questionnaires can easily be conducted on a large scale at district level (Lengeler *et al.*, 1991; Red Urine Study Group, 1995; Booth *et al.*, 1998) and for individual diagnosis in a given endemic setting (Ansell *et al.*, 1997; Utzinger *et al.*, 1998; Zhou *et al.*, 1998; Partnership for Child Development, 1999). A crude assessment of endemicity is possible using questionnaires administered by staff of the health or educational sectors. Self-reporting by schoolchildren was shown to detect 40% of light infections (<50 eggs/10 mL) and 80% of heavy infections (≥50 eggs/10 mL) (Chan *et al.*, 1998). Measured or perceived macrohaematuria and clinical signs and symptoms are good surrogate indicators of (heavy) morbidity.

All these methods are the basis of evaluation and monitoring in control programmes. These and future tests are particularly important in helping to detect early morbidity and its changes after treatment. In conjunction with visualizing methods such as ultrasonography, indirect measures should aim at predicting rather than merely reflecting functionally relevant and severe disease.

2. VISUAL METHODS TO ASSESS MORBIDITY DUE TO SCHISTOSOMIASIS

2.1. Available Methods

Schistosomal pathology can be demonstrated in individual patients by X-rays, computed tomography (CT), magnetic resonance imaging (MRI), endoscopy and biopsies (Forsyth and Macdonald, 1965; Bessa *et al.*, 1979; Dunn *et al.*, 1979; Aisen *et al.*, 1983; Patel *et al.*, 1993). However, these techniques can be performed only in a hospital setting. Ultrasonography was introduced in the 1970s as a safe, rapid, non-invasive and relatively inexpensive technique for assessing schistosomiasis-related lesions under hospital and field conditions. Its use has been validated by comparison with findings from biopsies, radiography and MRI (Abdel-Wahab *et al.*, 1978; Burki *et al.*, 1986; Scully *et al.*, 1994). The first study assessing schistosomal morbidity at community level (Degrémont *et al.*, 1985) generated a lot of further research. Ultrasound has since been widely used in surveys involving large numbers of people at community level (Doehring *et al.*, 1985a; Homeida *et al.*, 1988b, Nooman *et al.*, 1995). These field studies have included investigations of morbidity patterns in different geographical areas, and also follow-up studies after treatment (Hatz *et al.*, 1990a, 1998; Doehring-Schwerdtfeger *et al.*, 1992a; Ohmae *et al.*, 1992a, b). In assessing the size of the liver and the spleen, ultrasound was shown to be superior to clinical examination in areas where *S. mansoni* or

malaria or both are prevalent (Doehring-Schwerdtfeger *et al.*, 1992b; Kardorff *et al.*, 1997).

Ultrasonography does require considerable time, skill and experience, so the technique will not replace clinical and laboratory observations. The question of how far indirect indicators of morbidity – such as hepatomegaly or haematuria – can be used to assess damage to internal organs can be clarified by comparative studies using ultrasound in combination with other measurements (Burki *et al.*, 1986; Homeida *et al.*, 1988a; Abdel-Wahab *et al.*, 1989; Patel *et al.*, 1993).

2.2. Tools

Ultrasound scanning is available in more and more hospitals and clinics, and has greatly added to the possibilities for diagnosis and assessment of schistosomiasis morbidity in endemic areas. Portable ultrasound units which can provide reliable records of morbidity make it possible to extend the use of ultrasound to large-scale investigations in the field. The different machines available vary in the type of picture they provide so, for studies which assess standardized information, it is important to select one type of machine and probe for a given study, and use the same type throughout. This must be remembered in longitudinal studies. A device with both linear and sector probes gives maximum flexibility. Linear probes are suitable for examining the liver and spleen whereas a sector scanner is preferable for the detection of bladder pathology behind the symphysis. A curved linear probe represents a possible compromise for investigations of all types of lesions due to schistosomiasis (WHO, 1991).

Doppler ultrasonography is used in the evaluation of the haemodynamics of the portal circulation (Brandt *et al.*, 1995). Direction and velocity of flow are measured in portal, splenic and mesenteric veins. The technique facilitates the close follow-up of patients with portal thrombosis, and the monitoring of shunt functioning following surgery and of the regression of portal hypertension after sclerotherapy or after the introduction of portosystemic shunts (Magalhaes, 1986). The Doppler technique can be used to assess the severity of lesions due to hepatosplenic schistosomiasis in hospital-based patients (Abdel-Wahab and Mahmoud, 1987). Devices for field use are now becoming available (Kardorff *et al.*, 1999a).

2.3. Procedures

The ultrasonographic investigations and measurements made depend on the objectives of the examination. These may range from in-depth assessment of individual patients in hospital settings to cross-sectional screening at community level, examining several hundred people in order to obtain an

overview of prevalence and severity of lesions. In the former situation, a comprehensive evaluation of the abdominal organs, including Doppler sonography and possibly CT/MRI investigations is recommended for lesions due to *S. mansoni* and *S. japonicum*.

Pathological alterations due to schistosomiasis change substantially over time, mainly as a result of treatment. The key issues to understand are (i) the functional significance of the lesions and (ii) the state from which pathological lesions can still reverse, either spontaneously or after treatment. Reversal of lesions was first shown in infections due to *S. haematobium* and *S. mansoni* by Hatz *et al.* (1990a) and Doehring-Schwerdtfeger *et al.* (1992a) respectively after specific chemotherapy with praziquantel. Some pathological lesions seem to disappear spontaneously (Pugh and Gilles, 1979). However, relatively little is known about the functional significance of detected lesions and about the point at which pathological lesions become irreversible, or about the prevalence, progression and regression of pathology due to the *Schistosoma* species infecting humans among different age groups in different endemic areas. Thus, there are still substantial gaps in the information needed as a rational basis for efficient morbidity control strategies.

2.4. Standardization of Procedures and Training

Experience shows that severe pathology is relatively easy to assess (C.F.R. Hatz *et al.*, unpublished observations). There is more ambiguity with minor pathology, especially in *S. mansoni* infections, leading to more inter- and intra-observer variation in interpreting the recorded findings. It is important that methods should be refined so that early lesions can also be specifically and reproducibly detected, and the methods should be validated against a 'gold standard'. Patients with early stages of the disease will have the best chances of recovery. Furthermore, it can be expected that less severe pathology will be seen more often in endemic areas where successful control programmes are taking effect.

The patterns of schistosomal pathology and the way they vary in space and time can be assessed only if comparative studies are carried out in different endemic settings. Comparing the details of lesions in different epidemiological situations will be possible only if the examination procedures and the reporting of the results can be standardized.

It is vital to distinguish variations of findings in the target organs which are in the normal range from pathological lesions and determine which of these are specific for schistosomiasis. The normal range of size and appearance needs to be assessed in non-infected controls of the same age and sex from the same endemic area, because there is considerable variation. Organometric standards based on height and age have been defined and evaluated for populations of industrialized countries (Dittrich *et al.*, 1983). Only recently have such standards begun to

appear in the literature from endemic countries (Friis *et al.*, 1996; Yazdanpanah *et al.*, 1997; R.M. Olveda and H. Murakami, personal communications).

The interpretation of ultrasound findings is subject to intra- and inter-observer variation. This variance of interpretation can be minimized only by standardization. Experience shows that, even when ultrasonography is carried out by well-trained and experienced observers, there is some inter-observer variability, especially in reports of minor pathology (Doehring-Schwerdtfeger *et al.*, 1992c). There is also variation in the results of the same observer when observations are spread over a period of time, as in longitudinal studies (C.F.R. Hatz *et al.*, unpublished observation).

If the level of variation can be assessed – for example by replicate assessment by two or more different observers on the same patient – allowance can be made for it in evaluating the final results. However, variation among observers should be reduced as far as possible by careful training, and by quality control during surveys. The type of staffing required, and the goals of the training, need to be carefully defined according to the aims of the investigation. Investigators may be medical doctors or paramedical staff. Practical training with patients needs to be given by experienced ultrasonographers. Training needs to emphasize that the rigorous application of standard procedures not only for the ultrasound examination but also for the preparation of the patients is crucial (Cairo Working Group, 1992).

For medical doctors in endemic areas, training in detecting the particular lesions due to schistosomiasis should be part of a comprehensive course on the use of diagnostic ultrasound. Basic training in ultrasound use for general abdominal and specific obstetric indications is required for practitioners in rural hospitals. For medical and paramedical staff who are going to work specifically in schistosomiasis control programmes, training should focus on the important lesions due to schistosomiasis that can be unambiguously detected. Early pathology such as minor lesions of the bladder wall or signs of periportal thickening are often misclassified by inexperienced observers.

For quality control, close supervision during the surveys is mandatory, as are regular regional meetings with practical work on patients. Local investigators can discuss problems in such meetings that have arisen in the course of their practice. The moving image cannot be easily documented, and refesher courses using photographs alone will not be adequate.

3. SCHISTOSOME-RELATED MORBIDITY AS DETECTED BY ULTRASOUND

The three major *Schistosoma* infections (*S. haematobium*, *S. mansoni* and *S. japonicum*) present different pathologies. Even within one species, the pattern

of pathology tends to vary from one endemic area to another. These differences have been demonstrated in autopsy studies and by visualizing techniques, including ultrasound. Though the extent of variation is not yet clear, and may be affected by methodological factors, it is clear that pathology varies with exposure, age, sex, ethnic and geographical features, differences in host behaviour and the presence of concomitant diseases. The variation could also be due to intra-specific variation of the parasites and the intensity of infection.

Particular aspects of the use of ultrasound related to the pathology of all *Schistosoma* species are discussed in the following sections, summarizing present knowledge. A more extensive account of reported investigations up to 1991 has been published (Hatz *et al.*, 1992a, b, c).

3.1. *Schistosoma haematobium*

S. haematobium is a disease of the pelvis. The main organs affected are the urinary bladder, the ureters, the kidneys and the genital organs. The most common early lesions include granulomatous changes and ulcers of the bladder wall and the ureters leading to dilatation of the renal pelvis which can be seen in ultrasound imaging (Table 1). The term hydronephrosis is used to describe all stages of dilatation which result in impaired kidney function. The severity and frequency of pathological lesions and subsequent sequelae correlate with egg output and macro/microhaematuria, and also with the duration of the illness (Smith and Christie, 1986). A comprehensive account of the assessment and features of morbidity due to *S. haematobium* has been given by Chen and Mott (1989).

The first descriptions of urinary tract pathology using ultrasound were published in Egypt (Kenawi *et al.*, 1977; Abdel-Wahab *et al.*, 1978; Mongy *et al.*, 1978). The instrument used was a Sonograph III Greaton Scanner (Unirad Corporation) with a 3.5 MHz transducer at hospital level. Previously, Sanders and Bearman (1973) had demonstrated renal obstructive pathology due to causes other than schistosomiasis. Following the development of improved devices (real-time scanning), ultrasound became a well established tool in the evaluation of urinary tract diseases (Bartels, 1981).

Ultrasound is considered to be as sensitive as intravenous pyelography (IVP) in detecting bladder masses and stones (plain X-ray), hydronephrosis and renal stones, but detects hydroureter less frequently than IVP, and detects calcifications of the bladder wall and ureteral stones less frequently than X-ray (Burki *et al.*, 1986; Abdel-Wahab *et al.*, 1992a).

3.1.1. *Lesions of the Urinary Tract*

Pathological lesions of the lower and upper urinary tract are age-related (Hatz *et al.,* 1998; Traoré *et al.*, 1998), with a peak observed in the age group of 10–20 years in most studies. Standard views, preferably with a sector scanner, are recommended to increase reproducibility and to minimize inter-observer variation in interpreting the findings.

(a) *The bladder.* Adequate filling of the bladder is essential to avoid interpreting a normal appearance of the wall structure as pathological. Fluids (500–1000 mL) should be given half an hour to one hour before the examination, and subjects asked not to urinate during this period. In pregnancy, however, the enlarged uterus may not allow for adequate bladder filling.

Bladder pathology was shown to be consistently demonstrated by ultrasonography, compared with cystoscopy and intravenous pylography, especially on the posterior wall (Browning *et al.*, 1984; Degrémont *et al.*, 1985; Doehring *et al.*, 1985a; Burki *et al.*, 1986; Devidas *et al.*, 1988). However, it is conceivable that very early lesions may be missed. Lesions appear as irregularity or focal or generalized thickening ($\geq$ 5 mm) of the wall of a well filled urinary bladder.

Advanced disease presents with masses and granulomatous pseudopolyps protruding into the bladder lumen. Bladder wall calcifications are considered to be almost pathognomonic of schistosomiasis. An estimated number of 100 000 calcified eggs per cm^2 was considered to be detectable in a radiograph (Cheever *et al.*, 1975). Whereas gross calcification and bladder stones can be easily seen as echodense areas, causing a typical conical shadow in the ultrasound image (Heurtier *et al.,* 1986), minor to moderate calcifications detected by X-ray are often missed using ultrasonography (Burki *et al.*, 1986).

Schistosomal bladder lesions can be confounded with cystitis, amyloidosis, tuberculosis and bladder carcinoma (Pollack *et al.*, 1981). The latter may, however, occur together with schistosomal pathology and there is even some evidence of a relationship between carcinoma and earlier lesions resulting from schistosomiasis (Thomas, J.E. *et al.*, 1990; Mostafa *et al.*, 1995).

(b) *The ureters.* The dilated lower or upper end of the ureter is easily seen in cases with major dysfunction or obstruction (Dittrich and Doehring, 1986), but dilatation is often difficult to assess when only minor pathology is present. Thickening and wall irregularities and ureterocele-like lesions of the ureters were demonstrated in Malian children and adolescents (Kardorff *et al.*, 1994a).

However, ultrasound has limitations in examining changes in the ureters. Pyelography is clearly superior in detecting lesions of the ureter wall including calcifications (Hatz *et al.*, 1990a; Abdel-Wahab *et al.*, 1992a). The latter may even be seen on a plain abdominal X-ray image.

Schistosomal infection is heaviest in the lower third of the ureters and decreases towards the kidneys (Al-Ghorab, 1968). Autopsy studies show that

dilatation of the upper urinary tract in schistosomiasis is not necessarily due to obstruction of the ureters, but may result from functional incoordination or aperistalsis of the ureter (von Lichtenberg *et al.*, 1971). Sonographically undetected dysfunction or obstruction of the ureters would explain congestive changes of the kidneys in patients with no detectable bladder lesion, as granulomata of the kidneys are very rare (Abdel-Wahab, 1982). However, other causes of urinary tract obstruction must be carefully assessed in each endemic setting.

(c) *The kidneys.* Ultrasonography is the best method to demostrate kidney congestion, and is the easiest method to assess severe hydronephrosis leading to a non-functioning (silent) kidney. Even mild congestive changes are easily detected, and caution is required to avoid overestimating the importance of minor congestion that may be due to a full bladder (Morin and Baker, 1979; Hatz *et al.*, 1990b). Repeating the investigations when the bladder has been emptied is necessary in such instances to avoid false-positive results.

Kidney pathology is of important prognostic value, as renal failure can lead to death. Kidney congestion is not a unique pathognomonic sign of schistosomiasis, as other diseases can lead to such changes, including benign anomalies such as aberrant renal arteries (Pugh *et al.*, 1979).

Congestive changes of the kidneys have been classified by Ellenbogen and colleagues (1978) and reviewed by Weill and colleagues (1983). Five stages are proposed for the description of pelvis dilatation of the kidneys in children and adults: (i) fissure of pelvis; (ii) mild dilatation of pelvis; (iii) moderate dilatation of pelvis with slightly reduced parenchyma thickness (> 2 cm); (iv) severe dilatation of pelvis with narrow parenchyma border (< 2 cm); (v) end-stage dilatation of pelvis with absence of parenchyma. In small children, the ratio between the thickness of the parenchyma and that of the renal pelvis (2 : 1) is measured in the longitudinal diameter of normal-sized kidneys (Lutz and Meudt, 1984). Weill's grading system is currently recommended for use in classifying *S. haematobium* pathology (Cairo Working Group, 1992). Fissure and mild dilatation were classified as normal findings in the Niamey meeting in order to avoid ambiguity in assessing ultrasound imaging (Hatz *et al.*, 1998; WHO, in press). Dilatation of the renal pelvis may be influenced by the filling state of the urinary bladder. Therefore, it is essential to reassess the dilated renal pelvis after the bladder has been emptied.

3.1.2. *Lesions of the Liver and Spleen*

Lesions in the liver in *S. haematobium* and *S. mansoni* infections were reported in the early descriptions by Bilharz (1856) and Symmers (1904) and have also been recorded by Elwi and Attia (1962) in 10% of autopsies performed on adults infected with *S. haematobium*. Nooman and colleagues (1974) found

bilharzial granulomata in liver biopsies from almost half of a series of patients with hepatosplenic *S. haematobium* infections. Minor fibrotic lesions were found in sonograms of 40% of 200 adults living in an area where no case of *S. mansoni* infection had been reported (I. Ramzy, unpublished data). Nafeh and colleagues (1992) reported ultrasonographic changes of the liver in patients with *S. haematobium* infections. Significantly higher rates of periportal fibrosis in infected subjects (22%) than in subjects without apparent infection (12%) were found among 253 schoolchildren in a hospital-based study in Egypt. Up to 35% of infected schoolchildren were found to have mild periportal fibrosis in a community-based study (Abdel-Wahab *et al.*, 1992b). Furthermore, splenomegaly was found to be associated with the intensity of *S. haematobium* infection in this study.

Periportal fibrosis has not yet been reported in ultrasound studies of *S. haematobium* infection from geographical areas other than Egypt. A review of existing knowledge about potential fibrogenesis of *S. haematobium* concluded that periportal fibrosis is an unlikely event in this form of schistosomiasis (Eltoum *et al.*, 1993), based on observations in Sudan and on previous work in Egypt (Cheever, 1985). Liver lesions seen in individuals with *S. haematobium* infections may be due to undetected *S. mansoni* infections, but further study outside Egypt is needed.

3.1.3. *Geographical Variation in Observed Lesions*

Table 2 summarizes the pattern of morbidity as recorded by ultrasound in 13 selected studies conducted in nine African countries and correlates it with indirect measures of morbidity. The data are for subjects younger than 20 years of age, the age group with the highest prevalence of morbidity. Overall bladder and upper urinary tract pathology were chosen for comparison because they yielded the best retrievable figures.

The limitations of such a comparison are obvious. Data presented in the publications are based on different recording standards, especially before 1991 when the first guide for standard assessments was published by WHO. Most of the publications present data of cross-sectional surveys, and age and sex distributions varied considerably. However, no randomization of the population was done in any of the surveys. Approximate data were used for the Table when figures of acceptable precision for a particular age group could not be calculated precisely from existing information. Egg output and other indirect measures of morbidity were associated with ultrasound findings in all studies, but the exact figures were available in only some of them. No information for upper urinary tract pathology was available in the Egyptian study.

The range of prevalence of overall bladder pathology detected by ultrasound was from 28%, in a study from Mali, to 79% in Niger and Tanzania. The

Table 2 Summary of studies assessing urinary tract morbidity due to *S. haematobium*.

Country	No.	Infection		Age range examined (years)	Prevalence of bladder pathology (%)	Prevalence of upper urinary tract pathology (%)	Reference
		Prevalence (%)	Intensity[a]				
Tanzania	231	62	L	5–15	68	31	Degrémont *et al.*, 1985
Congo	213	10	M	3–80[b]	54	23	Doehring *et al.*, 1985a
Niger	273	69	L	5–14	71	2/19[c] 19	Heurtier *et al.*, 1986
Kenya	363	69	L	4–21	> 43[d]	14	King *et al.*, 1988
Cameroon	212	72	M	4–15	67	36	Gonsu-Fotsin *et al.*, 1989
Niger	130	91	H	?–15	79	36	Lamothe *et al.*, 1989
Tanzania	202	65	L	7–20	79	40	Hatz *et al.*, 1990a
Mali	408	65	?	6–15	28	38	Dabo *et al.*, 1995
Madagascar	184	76	L	6–14	62	10–20	Serieye *et al.*, 1996
Egypt	510	35	L	5–15	66	NA[e]	Medhat *et al.*, 1997
Mali	438	77	L	2–20	27	> 15[d]	Vester *et al.*, 1997
Tanzania	533	77	M	7–18	67	15	Hatz *et al.*, 1998
Ghana	579	57	M	5–14	>46[d]	> 7[d]	Wagatsuma *et al.*, 1999

[a] Most commonly used egg count classes (differing from WHO classification): L = low (<100 eggs/10 mL urine), M = moderate (100–350/399 eggs/mL); H = high (>350/399 eggs/mL).
[b] 17 adults included in the study.
[c] Girls/boys.
[d] Approximate values.
[e] Not available.

figures for upper urinary tract pathology ranged between 2% (girls in Niger) and 40% in Tanzania. As expected, pathology of the bladder was consistently commoner than lesions of the upper urinary tract, except in a study from Mali. The authors of that study used the common classification by Weill and colleagues (1983) for the assessment of renal lesions. So it is possible that mild congestive lesions were overestimated, but this cannot be concluded from the presented data, as no detail was given.

No obvious difference in morbidity patterns between the five East and six West African studies was detected. Intensity of infection was consistently associated with severity of lesions (data not shown). Although no conclusion can be drawn from the existing data sets with regard to severity of the lesions, the information provided does not indicate a major difference between West and East African settings such as that postulated for morbidity due to *S. mansoni* (Kardorff *et al.*, 1996).

This attempt to make a comparison clearly shows that data from different endemic settings should be presented in a more standardized way in order to render more accurate comparisons possible. This will help in reaching more general conclusions for key control issues such as treatment and re-treatment schedules.

3.1.4. *Evolution of Lesions*

The first reports on reversibility of uropathy after treatment were based mainly on X-ray studies in hospitals and gave contradictory results (Forsyth and Bradley, 1964; Lucas, *et al.*, 1966; Davis, 1966; Lehmann *et al.*, 1973; Farid *et al.*, 1976). Some of the lack of uropathy resolution may have been due to the lower efficacy of the drugs used in the earlier studies. Since then, more potent drugs have become available, and the availability of ultrasound has made it feasible to investigate the development more consistently. Doehring and colleagues (1985b) found clearance of bladder lesions one month after a single oral dose of praziquantel (40 mg/kg body weight) in schoolchildren, and 69% of bladder lesions and 73% of renal lesions were found to resolve within 10 months in a cross-sectional study in Niger (Devidas *et al.*, 1989). The authors found that older subjects had significantly less reduction of renal lesions than children aged between 5 and 9 years. King and colleagues (1988) reported significant reductions in bladder lesions but no significant change in kidney pathology among 547 schoolchildren re-examined one year after praziquantel treatment in Kenya.

A cohort of 194 children was also subsequently re-examined 7 to 13 years after the initial surveys and after five annual treatment rounds, and compared with a group of previously untreated subjects. Bladder wall morbidity was 11-fold lower in treated subjects than in the untreated control children and severe

hydronephrosis was completely reversed. Urinary tract lesions were thus significantly reduced despite reinfection, suggesting that cumulative intensity and duration of infection during early adolescence were important risk factors for morbidity (Subramanian *et al.*, 1999).

In Tanzania, Hatz and colleagues (1990a) reported clearance rates of 90% for bladder and kidney lesions among schoolchildren within 6 months after treatment with praziquantel. All pathology clearances correlated with a rapid reduction of egg output.

Resolution of lower urinary tract pathology was found to be nearly 100% within a few months after treatment among the population of eight villages in rural Ghana (Wagatsuma *et al.*, 1999). However, 69% of upper urinary tract pathology was not entirely resolved by 18 months. Younger subjects cleared the lesions more rapidly than older subjects.

Prevalence of vesico-ureteral reflux and of pyelo-caliceal reflux had decreased by 96% and 78% respectively 12 months after praziquantel treatment among 23 and 54 individuals in a study in Madagascar (Rasendramino *et al.*, 1998). Re-examination of 104 Malian subjects who previously had ureteric dilatation showed no evidence of pathological lesions one year after standard treatment with praziquantel (Kardorff *et al.*, 1994a). Post-furosemide urography (Sharafi and Rayis, 1989) and diuretic scintigraphy (Bahar *et al.*, 1990) demonstrated the lack of urodynamically relevant obstruction in patients with upper urinary tract pathology due to schistosomiasis. This may explain the observed reversibility of such lesions after chemotherapy without residual renal impairment.

A study in Tanzania (Hatz *et al.*, 1998) investigated the evolution of pathology during 24 months after chemotherapy, and the severity and the incidence of reappearance of lesions among a cohort of 224 children living in an area of moderate to high transmission of *S. haematobium*. The proportion showing lesions was 76% at the start of the study and decreased sharply after treatment to 11% at 6 months. At 24 months, lesions were detected in 57%, and 11% had developed new severe pathology. Children with severe pathology at baseline developed new severe pathology significantly more often than those without, or with mild, pathology at baseline. On the other hand, development of moderate or severe pathology was recorded significantly more often among children with no lesion at baseline than among those with reappearance of lesions after clearance. These apparently conflicting findings raise two questions (i) to what extent may predisposing factors enhance the development of pathology in severely affected children? and (ii) to what extent are children who have recently cleared schistosomal lesions protected from severe pathology?

In a study in Ghana with an almost identical design, 9·5% of the subjects with bladder lesions at the start of the study showed them again when re-examined at 18 months. Only two subjects had a bladder wall mass or polyp. Subjects who had pre-treatment bladder pathology had a higher risk of developing

bladder lesions at 18 months (Wagatsuma *et al.*, 1999). The difference between the two studies was attributed to the different transmission patterns of the disease.

Reinfection risk decreases with age and increases with exposure and pretreatment intensity (Etard *et al.*, 1995; C.F.R. Hatz *et al.*, unpublished observations). Age-acquired resistance to reinfection is mirrored in longitudinal studies on urinary tract morbidity. In areas of moderate endemicity in Mali and in Madagascar, Serieye *et al.* (1996) and Vester *et al.* (1997) demonstrated a decrease in lesions of both the lower and the upper urinary tract with increasing age. The decrease was lowest in patients older than 40 years. This age distribution was less marked in cross-sectional studies from high transmission areas on two Tanzanian islands, where pathology was found to be highest among the 15–19-year-old males, but where the prevalence among the older age groups was not significantly lower (Forsyth and McDonald, 1965; Hatz *et al.*, 1990b).

In an area of moderate to high transmisssion in mainland Tanzania, children with severe pathology were found to be more likely to develop new lesions 18–24 months after chemotherapy, indicating a higher risk (Hatz *et al.*, 1998). The number of children with severe pathology rose to pre-treatment levels of more than 10% within 24 months after initial treatment with a standard dose of praziquantel. However, no exposure data were collected among these children. In an area of low to moderate endemicity in Ghana, both age and water contact patterns were factors in the recurrence of urinary tract pathology (Wagatsuma *et al.*, 1999).

Based on the experience in different endemic settings, it is concluded that re-treatment will not be necessary earlier than 12 months or even several years in order to prevent the development of severe pathology (WHO, in press). The observation that reappearance of pathological lesions is associated with the intensity of pretreatment infection and the level of morbidity, as assessed by ultrasound, could indicate that some individuals are predisposed to developing lesions. This raises the question of a genetic factor controlling susceptibility/resistance to *S. haematobium* infection, which will have to be established by studies on genetic markers (Abel and Dessein, 1997).

3.1.5. *Genital Schistosomiasis*

Schistosomal lesions of the female and male genital tracts due to *S. haematobium* (few cases have been reported for other schistosome species; Qunhua *et al.*, 1997) have been underestimated over many years despite the fact that they were first reported a hundred years ago (Madden, 1899, 1911). Autopsy studies in Africa have since documented the presence of lesions of the lower and upper reproductive tracts (Charlewood *et al.*, 1949; Gelfand *et al.*, 1971;

Edington *et al.*, 1975; Wright *et al.*, 1982). Reports on genital schistosomiasis in travellers not living in endemic areas have also been published (Corachan *et al.*, 1994; Blum *et al.*, 1998). The reasons for underdiagnosis are manifold. Symptoms and signs are not reported or confounded with those of sexually transmitted diseases, diagnosis is difficult in rural health facilities of endemic areas, and medical expertise is lacking in both endemic and non-endemic countries.

(a) *Female genital schistosomiasis.* Studies in Tanzania and Madagascar indicate that *S. haematobium* causes lesions of the upper and lower genital tract of women in endemic areas (Leutscher *et al.*, 1998; Poggensee *et al.*, 1998). The Tanzanian study showed that conventional infection assessment based on a single egg count misses 31% and single reagent strip tests miss 57% of women with genital lesions (Poggensee *et al.*, 1998). Recognizing genital schistosomiasis, which may affect 9–13 million women in endemic areas of Africa (Feldmeier *et al.*, 1995), appears to be important for numerous reasons. Lesions of the genital tract are associated with gynaecological symptoms (G. Poggensee, personal communication), may lead to infertility in women, and may represent risk factors for sexually transmitted diseases including human immunodeficiency virus infection. However, the epidemiological evidence is so far lacking. Genital lesions among women apparently develop at a later stage than those of the urinary tract, when intensity of infection as measured by urinary egg output decreases. Development of lesions may be triggered by vascular changes in the pelvic area during puberty and pregnancy and is possibly influenced by hormonal factors (Feldmeier *et al.*, 1998).

Lesions of the lower reproductive tract are assessed by direct clinical methods such as colposcopy. Pathological lesions of the upper reproductive tract are more difficult to assess. Transabdominal ultrasound has been applied in preliminary studies in Malawi and in China (S.Z. Liu, personal communication), showing enlargement of the ovaries, the corpus and the cervix uteri, ovarian and uterine hyperechogenicities and one palpable hyperechogenic mass of the adnexes (Richter *et al.*, 1995). The authors concluded that the true prevalence of lesions of the upper reproductive tract may be underestimated by transabdominal ultrasound. Studies using the more sensitive transvaginal ultrasound are therefore planned.

(b) *Male genital schistosomiasis.* Eggs were demonstrated in the ejaculate of 43% of male subjects aged 15–49 years in an area endemic for *S. haematobium* in Madagascar (Leutscher *et al.*, 2000). Significantly reduced ejaculate volume and a higher rate of oligospermia were found among the few subjects with genital lesions compared with those without. In a post-mortem study in Egypt, the egg load in the seminal vesicles was found to be higher than that in the upper part of the ureter (Smith *et al.*, 1974). Despite a high estimated prevalence of genital schistosomiasis in male subjects

infected with *S. haematobium*, lesions causing dysfunction are considered to be exceedingly rare (Gelfand *et al.*, 1970) and unlikely to cause infertility (Patil and Elem, 1988). However, no case-control or prospective study exists which clarifies the issue.

Abnormalities of the prostate and seminal vesicles have been demonstrated using ultrasound in Madagascar (Leutscher *et al.*, 2000). Transrectal sonography was performed on nine White travellers infected with *S. haematobium* (5), *S. intercalatum* (2), *S. mansoni* (1) and mixed *S. haematobium/S. mansoni* (1), who had complained of haemospermia (Vilana *et al.*, 1997). The most frequently demonstrated lesions were calcifications of the prostate (n=7). A few cases of seminal vesicle calcification, hyperechogenic foci of the prostate, and dilatations of the seminal vesicles and the ejaculatory ducts were demonstrated. Some of the lesions regressed within 3 months to 6 years after treatment with praziquantel. More information from infected subjects in endemic areas is clearly warranted in order to assess the importance of male genital schistosomiasis.

3.1.6. *Key Issues of Morbidity due to* S. haematobium

Pathological lesions of the urinary tract develop rapidly among a high proportion of school-aged children living in endemic areas. Bladder lesions are highly associated with egg output and other markers of morbidity. Resolution or regression of all lesions within 6 to 12 months after specific treatment with praziquantel is normally seen. Re-treatment in areas of continuing infection is recommended for children and adolescents between once a year and every 3 years, depending on transmission and exposure intensities and the initial endemic level. Evaluation of control activities, including the use of ultrasound among sentinel groups, will determine the periodicity of treatment.

More epidemiological studies on genital schistosomiasis are warranted in order to define its public health importance.

3.2. Schistosoma mansoni

3.2.1. *Lesions Seen in Intestinal Schistosomiasis*

Infection with *S. mansoni* results in granulomatous lesions of various organs, including the liver, the portal vein and its tributaries, the spleen and the gall bladder (Table 3). Alterations of the intestinal wall have been documented using hydro-ultrasonography (Dittrich *et al.*, 1994) as has

Table 3 Development of *S. mansoni*- and *S. japonicum*-related pathology and its reflection in trans-abdominal ultrasound imaging in endemic areas.

Pathology	Pathognomonic lesions in endemic areas[a]	Ultrasound correlate of pathology	Detectable by ultrasonography (sensitivity)[a]
Liver			
Periportal thickening (fibrosis)[b]		Echodense areas along the portal vein, best seen in left liver lobe	
Early thickening	+	Scattered echodense areas	(+)/+
Moderate thickening	+	Echogenic bands	++
Advanced thickening ('pipe-stem')	+++	Tubular and confluent echogenic patterns	+++
'Network' pattern[c]	++	Lobular nets	++
Granuloma in liver texture[c]	(+)	Echodense mass unrelated to portal tree	++
Portal vein			
Diameter of lumen enlarged[d]	(+)	Standards related to height	++
Presence of collateral veins (adults)	(+)	Coronary, paraumbilical, short gastric veins	++
Gall bladder			
Wall thickening	(+)	Increased echodensity of thickened wall	++
Spleen			
Congestive splenomegaly	-	Standards related to height	+++
Ascites	-	Free fluid in abdomen	++

[a] –, Not pathognomonic, not detectable by ultrasonography; (+), limited pathognomonic specificity or detectability by ultrasonography; + to +++, increasing degrees of good pathognomonic specificity and detectability by ultrasonography.
[b] Thickening in this area gives rise to an increased width of echodensity around the portal tree. The value obtained is the average of the outer-to-outer measurements of two first-order segmental branches, from which the diameter of the lumen has been subtracted.
[c] *S. japonicum* infections.
[d] The internal (inner-to-inner) diameter of the portal vein is measured at the entry point of the portal vein into the liver.

schistosomiasis-related pulmonary hypertension using echocardiography (Emanuel *et al.*, 1987).

(a) *The liver.* The hepatic lesions affect mainly the portal tree, leading to periportal fibrosis and eventually to portal hypertension and oesophageal varices. Early lesions such as patches of fibrosis are often focal, and are more reliably detected by ultrasound scanning than by biopsies, because the whole liver can be scanned. However, subtle inflammatory changes may not be detected using ultrasound, as was shown in a comparative appraisal with magnetic resonance imaging (Patel *et al.*, 1993). Unfortunately, this technique is not available for field surveys.

Concomitant cirrhosis due to other diseases may confound the assessment of periportal thickening indicating periportal fibrosis due to schistosomiasis in ultrasound examinations (Pereira *et al.*, 1998).

The development of portal hypertension as a result of periportal fibrosis leads to the development of oesophageal varices. Bleeding from such varices is the usual cause of death due to *S. mansoni* infections. In hospitals, Doppler sonography can be used instead of angiography to assess the degree of portal hypertension (Abdel-Wahab and Mahmoun, 1987).

The first reports on liver pathology using ultrasound described echogenic areas of thickened, fibrosed portal tracts in the liver of infected patients (Abdel-Latif *et al.*, 1978; Abdel-Wahab *et al.*, 1978). The use of ultrasound to measure the diameter of the portal vein was first reported by Carlsen and Filly (1976). When collateral veins become large enough to be detected by ultrasound this already indicates enlargement. Coronary, paraumbilical and short gastric veins are the ones usually detected.

Left lobe hypertrophy (Mackenjee *et al.*, 1984) and atrophy of the right lobe are ascribed to higher vascular flow in the left lobe (Cerri *et al.*, 1984; Mies *et al.*, 1985). In advanced disease, however, the liver is usually normal in size (Abdel-Wahab *et al.*, 1978, 1989) or even diminished (Fataar *et al.*, 1984).

Presinusoidal periportal thickening is more frequently detected in ultrasound scanning than are granulomatous changes in the liver texture (Cerri *et al.*, 1984). The echogenic picture of the pathognomonic 'clay pipe-stem' fibrosis consists of a double echogenicity representing the walls of the portal vein branches, separated by a translucent lumen (Hussain *et al.*, 1984). Such changes are usually seen at the bifurcation of the portal vein, but peripherally located fibrotic changes around the portal tree were also found in children with early stages of the disease (Doehring-Schwerdtfeger *et al.*, 1989). Homeida and colleagues (1988a) proposed a classification of periportal thickening which has subsequently been used by many investigators (Zwingenberger *et al.*, 1989; Davidson *et al.*, 1991) as follows. Grade 1: minimal echogenic thickening of the walls of two or more portal radicles (<3 mm) with little change in the diameter of the main portal vein wall); grade 2: mild echogenic thickening of the walls of two or more portal vein radicles (3–5 mm), mainly peripherally,

with little or no thickening of the wall of the main portal vein; grade 3: moderate to severe periportal thickening of most portal vein radicles (>5–7 mm) with marked narrowing of the central lucency, marked thickening at the bifurcation of the portal vein and extending to the surface of the liver, and at the wall of the main portal vein; grade 4: marked thickening of the walls of the portal vein radicles with obliteration of the central lucency in the peripheral branches forming thick echogenic bands ranging in thickness from >7 to 20 mm. Further classifications were developed and adjusted (Abdel-Wahab *et al.*, 1989; Doehring-Schwerdtfeger *et al.*, 1989).

Ultrasonographic evaluation of periportal thickening is complex, particularly because the early signs are not specific and because it is difficult to define the locations where thickening should be measured. Therefore, an expert meeting held in Niamey (WHO, in press) agreed to compare two methods of assessing periportal thickening in different endemic settings. The first one is based on classical, standardized measurement of periportal thickening. The second, novel approach assesses the periportal thickening according to a set of image patterns of the liver texture. It has the advantage that it incorporates the texture of the entire liver, but it may be subject to increased inter-observer variation due to a lack of standardization. On the other hand, the classical measurement of periportal thickening provides standardized information, but can give misleading results, since pathological changes will not be recorded if they do not occur at the standardized focal points where the measurements are conducted. The two methods proposed in the protocol (WHO, in press) will be assessed in the light of future experience when comparative data from different endemic areas are available.

The classifications given by Homeida *et al.* (1988a) and Doehring-Schwerdtfeger *et al.* (1989) differ with regard to the definition of which type of periportal thickening was considered 'early' and which 'advanced'. While the former suggested that initial changes were seen in the periphery and that central fibrosis was a sign of advanced disease, Doehring-Schwerdtfeger and colleagues (1989) described lesions of the portal vein and its bifurcation as initial stages, while peripheral periportal lesions were attributed to more advanced stages. In the first workshop on the topic (Cairo Working Group, 1992), Abdel-Wahab *et al.*'s (1989) classification, which excluded central lesions from the staging process, was finally agreed upon as the standard to be further tested. Technically, the thickness of portal branches is measured from outer to outer wall, and the diameter of the main portal vein is measured from inner to inner wall, mid-way between the porta hepatis and the bifurcation.

Gerspacher-Lara and colleagues (1997) also contributed to this discussion. In a Brazilian study population (mean age 32 years, range 1–86 years; prevalence of 66% *S. mansoni* infections detected with two Kato–Katz thick smears) they attributed the subjects to one of three groups with central, peripheral or mixed central/peripheral thickening of the periportal areas. Central thickening

was associated with the presence of peripheral thickening. Splenomegaly, which they considered the 'hallmark of advanced disease', was found in 16% and 15% of subjects with peripheral and mixed periportal thickening, respectively. No case with central but no peripheral thickening was found. Based on their findings in Brazil, these authors concluded that central periportal thickening occurs more frequently among older subjects but should not be considered a criterion for advanced disease.

Medhat and colleagues (1998) cautioned that hepatic periportal thickening may be caused by prolonged pyrexia also. They found grade II lesions (WHO, 1991) in 3% of Egyptian patients without previous exposure to schistosomiasis. Their findings need to be validated by other groups and in other settings.

An association of oesophageal varices with enlarged diameters of the portal and splenic veins in patients with *S. mansoni* infections has been found by investigators in different endemic settings (Abdel-Latif *et al.*, 1981; Richter *et al.*, 1992). Cases with minor periportal thickening had oesophageal varices but no bleeding. M.A. Homeida (personal communication) found that 4% of patients with periportal thickening had haematemesis during a 3-year follow-up period. In contrast, Davidson and colleagues (1991) found no correlation between the grade of periportal thickening and the severity of oesophageal varices in Zimbabwean adults. Periportal thickening, a splenic longitudinal dimension > 11 cm (not height-dependent) and varices were found to be independently associated with a risk of variceal bleeding among Sudanese subjects in a case–control study (Eltoum *et al.*, 1994). Similar results were reported from Brazil (Domingues *et al.*, 1993). Scores of various liver lesions, including periportal thickening, portal vein diameter, spleen size and porta-systemic anastomoses were evaluated to assess retrospectively the risk of bleeding from oesophageal varices (Abdel-Wahab *et al.*, 1993). A prospective study over 4 to 65 months among 27 Brazilian patients showed that sonographic scores based on the degree of periportal thickening and portal vein diameter at baseline had a predictive potential for bleeding from oesophageal varices (Richter *et al.*, 1998). The authors of this study suggested that ultrasound could be used to identify high-risk patients at peripheral health care level in endemic areas, and application of endoscopy conducted at central level could then be restricted to patients at risk of future oesophageal bleeding.

Doppler sonography was introduced by Abdel-Wahab and Mahmoud (1987) for the evaluation of the haemodynamics of the portal circulation in hepatic schistosomiasis. Direction and velocity of flow are measured in portal, splenic and mesenteric veins. In contrast to the situation in cirrhosis (Zoli *et al.*, 1986), the velocity of the portal flow is increased in up to 80% of schistosomiasis patients with portal hypertension (G.G. Cerri, personal communication). Colour Doppler sonography may be useful in assessing the evolution of portal hypertension after treatment (Texeira Brandt *et al.*, 1995).

Liver cirrhosis is easily differentiated from schistosomal lesions sonographically, because it typically affects the texture of the entire liver including

the lobuli, and causes surface nodularity and increased size of the caudate lobe (Abdel-Wahab *et al.*, 1989). Further possible confounders of liver pathology include leukaemia and lymphatic infiltrations and other metastatic cancers (Hussain *et al.*, 1984; El-Rooby, 1985), vinyl chloride toxicity (Doehring-Schwerdtfeger *et al.*, 1989) and Gaucher's storage disease (Glass *et al.*, 1987).

(b) *The gall bladder.* Thickening of the gall bladder wall and the gall bladder neck have been reported by several authors (Cerri *et al.*, 1984; Ali *et al.*, 1990; Doehring-Schwerdtfeger *et al.*, 1990). Reports on the effect of schistosomal damage to the gall bladder on the development of cholelithiasis are controversial (El-Hawey *et al.*, 1989a; Richter and Feldmeier, 1991).

(c) *The spleen.* Spleen enlargement can easily be detected by ultrasound and is found in almost all patients with periportal thickening (Cerri *et al.*, 1984; Abdel-Wahab *et al.*, 1989). Abdominal palpation of the spleen is inferior to ultrasound for the assessment of splenomegaly and may be a reason for conflicting conclusions in the literature about schistosomiasis and other causes of spleen enlargement (Gerspacher-Lara *et al.*, 1998).

Splenomegaly appears to parallel the severity of liver involvement (Homeida *et al.*, 1988b). However, in areas where malaria is endemic, the assessment of splenomegaly may not be a valuable indicator for schistosomiasis.

3.2.2. *Evolution of Lesions*

In the first published report using ultrasound in a follow-up of adult patients infected with *S. mansoni* who had been treated with praziquantel, El-Hawey and colleagues (1989b) found a reduction of enlarged portal vein diameters. Homeida *et al.* (1991) confirmed these findings in Sudan.

Histologically, fibrosis occurs when matrix formation is stimulated by parasite egg products. When these stimuli decrease, the same cell type stimulates fibrolysis leading to a gradual decrease in fibrosis (Andrade and Peixoto, 1992). Ali and colleagues (1991) found a higher resolution of periportal pathology 7 months after treatment in children aged under 11 years than in older ones, indicating a lower degradation potential in long-standing lesions. Detailed studies by Doehring-Schwerdtfeger and colleagues (1990, 1992a) showed a marked shift from high to low degrees of periportal thickening among Sudanese children within 23 months after treatment. Other studies have demonstrated the reversal of periportal thickening (Homeida *et al.*, 1988c, 1996). A 3-year follow-up study with yearly chemotherapy among 283 villagers in the central highlands of Madagascar found a marked decrease of the overall prevalence of periportal thickening, from 28% in the first year to 10% in the third (Boisier *et al.*, 1998). Most subjects had already cleared their lesions and all five cases with major periportal thickening (grade III) improved after two treatment rounds. The reduction of hepatosplenic morbidity at

community level may be achieved by at least three mass treatment courses plus the provision of praziquantel for treatment of individual cases (Homeida *et al.*, 1996). Children receiving an initial single dose of praziquantel had a similar reduction of morbidity after 2–5 years as those receiving two treatments at 1 and 2 years of the study period (Frenzel *et al.*, 1999).

The enlarged, height-adjusted portal vein diameter may decrease more rapidly in individuals with pathology than the periportal thickening, indicating that this is a potentially useful measure of functional pathology resolution (J. Richter, personal communication).

3.2.3. Schistosoma *Infection in a Newly Exposed Population: the Case of Richard-Toll in Northern Senegal*

In 1986 a dam to block the intrusion of salt water from the sea became operational on the coast of Senegal. Shortly before this event, an irrigation system for sugar cane plantations was upgraded near Richard-Toll, a town some 100 km upstream. These changes apparently led to an increase of *Biomphalaria pfeifferi* snails from a nearby lake and subsequently to *S. mansoni* infections among the population of these areas. The first case of *S. mansoni* infection was recorded in January 1988 (Talla *et al.*, 1990). Two years later, a survey conducted in the area revealed a prevalence of 60% for *S. mansoni* and 1% for *S. haematobium* among the local population. Ultrasound surveys of pathology related to *S. mansoni* were conducted in late 1990 (Rouquet *et al.*, 1993), in the autumn of 1993 (Kardorff *et al.*, 1996; Yazdanpanah *et al.*, 1997), in June 1995 (Lanuit *et al.*, 1996), and in July 1996 (Burchard *et al.*, 1998). These studies, conducted 3, 5, 7 and 8 years after the first cases of *S. mansoni* infection, provided a unique opportunity to assess the onset of early development of hepatosplenic morbidity in an untreated population (Thomas *et al.*, 1997). In Richard-Toll, an area with very high intensity of infection, 33% of 358 subjects were found to have periportal alterations in 1990, but only one person had a significant alteration; 11% had enlarged portal vein diameters. Only mild or moderate, but no severe, periportal thickening was recorded among 613 subjects from a village near Richard-Toll in 1993. Some of the subjects had been previously treated with praziquantel, but even when these subjects had high intensity infections they showed fewer increased periportal diameters than untreated subjects. This indicates either that the lesions had resolved after treatment and the periportal diameter had not had time to increase again, or that the previous treatment had a protective effect against the development of more severe lesions. The relatively low level of morbidity may have had many causes. A time lag of 5 to 10 years between the peak frequency of heavy infections in adolescents and the occurrence of periportal fibrosis in young adults was demonstrated by Homeida and colleagues (1998b) in Sudan. Thus, the

earliest detectable lesions would be expected at just about the time of these surveys, even in subjects with high intensity infections. Incidence, density of infections, parasite strain and host factors may also have influenced the low level of morbidity.

Mass treatment was started in 1993 in most affected villages and may have contributed to limiting the alterations of the periportal areas of the liver, although parasitological cure rates following single-dose praziquantel treatment were low (Stelma *et al.*, 1995). This unprecedented lack of efficacy raised a number of questions. It could have been due to repeated infections of very high intensity, the maturation of pre-existing prepatent *S. mansoni* infections, and possibly to a strain-specific reduced susceptibility to praziquantel (Gryseels *et al.*, 1994). Interestingly, commonly observed levels of efficacy could be achieved by two treatments with praziquantel at a dose of 40 mg/kg, given 40 days apart (Picquet *et al.*, 1998).

The lack of severe hepatosplenic lesions, as seen in Richard-Toll, was also reported from a long-standing focus in Mali (Kardorff *et al.*, 1994b). Parasite strain differences, undetected concomitant infections and unknown toxins or immunogenetic factors may contribute to the observation that more pathology is recorded from Egypt, Sudan, Zimbabwe and East African countries than from West African foci. The reasons for these geographical differences merit further investigation.

The low prevalence of organomegaly recorded in the first surveys in Richard-Toll in 1990 and 1993 is puzzling. Hepatomegaly of infected individuals was initially comparable to that in a control group in a non-infected area. Splenomegaly was found in 30% in an area of malaria endemicity (parasite rate of *Plasmodium falciparum*: 5%). The surveys of 1995 and 1996 showed higher rates of left liver lobe enlargement (infected area: 91–100%; non-infected area: 75%), but comparable rates of spleen enlargement (33%; 11–25%). Liver and spleen enlargement is considered to be an acute or subacute inflammatory reaction in heavily infected children and adolescents. An increase in malaria cases (numbers not stated) had been recorded since 1993, which makes the relatively low splenomegaly rates even more puzzling (Stelma *et al.*, 1997).

S. haematobium is also prevalent in the area. Seven years after the completion of the dam construction, an increase in numbers of *S. haematobium* infections was noted in four villages of the middle valley of the Senegal river basin when an ultrasound investigation was conducted among the resident population before and 4 months after treatment (before the transmission season) with praziquantel (Delegue *et al.*, 1998). Some of the individuals had been treated 23 months previously with a standard dose of praziquantel; 40% and 2% of the 203 subjects had lesions of the urinary bladder and the upper urinary tract, respectively, before treatment and 11% of the 182 subjects had bladder lesions at 4 months after treatment. Bladder irregularities were found

in 10 of 200 subjects from four villages in a non-endemic area. The authors concluded that *S. haematobium*-related morbidity can develop rapidly even in an area where a relatively modest increase of endemicity has occurred. This observation corresponds to the findings in an area of moderate endemicity, in which reappearance of morbidity was documented 12–18 months after treatment and clearance of pathological lesions (Hatz *et al.*, 1998).

Long-term monitoring of cohorts from different endemic settings of the Senegal river basin for schistosomiasis-related morbidity will greatly contribute to the further understanding of the evolution and dynamics of schistosomiasis-related pathology among the population of these new foci.

3.2.4. *Acute Schistosomiasis*

Acute schistosomiasis has been reported among travellers to endemic areas and among Brazilian patients. Its sonographic features have been described in various papers (Lambertucci *et al.*, 1994; Rabello *et al.*, 1994; Cesmelli *et al.*, 1997). Liver and spleen enlargement and hypoechoic nodules of the liver texture were reported in these patients, who had not previously been exposed to *S. mansoni*. Enlarged lymph nodes surrounding the portal vein, some of them with hyperechogenic central regions, were the most common features among children. Pyogenic liver abscesses were seen in three children, indicating that acute schistosomiasis favours the colonization of the liver by bacteria (Texeira *et al.*, 1996). Resolution of all pathological lesions was seen in adults, but only incomplete regression was noted in children (Cesmelli *et al.*, 1997; Rabello *et al.*, 1997).

Acute schistosomiasis has not been reported from African foci of either *S. haematobium* or *S. mansoni* infections (C.F.R. Hatz and M. Corachan, unpublished observations).

3.2.5. *Key Issues of Morbidity due to* S. mansoni

A prominent feature of morbidity is the time lag of 5 to 10 years between the peak intensity and frequency of infection, and the occurrence of periportal fibrosis or signs of portal hypertension among endemic populations. As a result of this time lag, the pathological lesions detected in surveys are not always associated with egg output. The demonstration of key liver pathology (periportal thickening) using ultrasound is superior to liver biopsies because the lesions have a patchy distribution. However, the standardized assessment of periportal lesions is still a matter of debate among experts and needs clarification in further studies. There are also granulomatous changes which are not readily detected by ultrasound (e.g. in the intestinal wall).

Sonographic scores may be helpful in predicting who will be likely to suffer from oesophageal bleeding. A marked but not complete resolution of liver lesions is age-dependent and occurs gradually over 7 months to 3 years after repeated treatment with praziquantel at a rate related to the initial pathology. Multiple treatment rounds are necessary to achieve sustained reduction of morbidity. In areas of continuing infection, re-treatment may be necessary once a year or even more frequently, depending on levels of transmission, exposure and, possibly, the susceptibility of the local parasite strains.

3.3. *Schistosoma intercalatum*

Only one publication refers to ultrasound investigations in infections due to *S. intercalatum*. A study in northern Uganda revealed the presence of this schistosome in six of 636 schoolchildren with low intensity infections (Odongo-Aginya *et al.*, 1994). Except for ultrasonographically confirmed splenomegaly in three cases, no morbidity was apparent. Periportal thickening of the periportal segments of the liver was not observed. There has been no report yet of ultrasonographically detected morbidity due to *S. intercalatum* from other endemic areas such as West and Central African countries, though prevalences of infection there are higher.

3.4. *Schistosoma japonicum*

Clinically, morbidity induced by *S. japonicum* is more severe than that due to *S. mansoni* in terms of liver pathology. The daily egg output is 20 to 50 times higher in the former, leading to a high incidence density and therefore to higher morbidity. This leads to a different age–prevalence profile in endemic settings in China. A high rate of early morbidity is found in young children. In older age groups, who have been exposed to infection over a prolonged time, the high level of morbidity contrasts with relatively low egg output (Anonymous, 1997). Many of the hepatic lesions due to *S. japonicum* infection are similar to those caused by *S. mansoni*. However, there are a few lesions that are characteristic of *S. japonicum* infections, particularly at advanced stages (Murakami, 1986; Ohmae *et al.*, 1992a; Yi and Wong, 1992).

The detection of lesions due to *S. japonicum* infection using ultrasound was first described by Wu in 1962. The method has since been used by many investigators in both hospital practice and field studies (Tang, 1986; Cai, 1987; Ohmae *et al.*, 1992a; Wiest *et al.*, 1992). A considerable body of knowledge on *S. japonicum*-related morbidity exists in the Chinese literature which is only partly accessible to the international research community (Cai *et al.*, 1992).

3.4.1. *Lesions Seen in the Liver*

Periportal thickening, enlargement of the portal vein and other hepatosplenic effects are common to both *S. japonicum* and *S. mansoni* infections. With *S. japonicum*, there is a series of characteristic changes in the liver parenchyma. The observed changes probably correspond to alterations in the liver architecture due to the development of areas of fibrosis (Cai *et al.*, 1992). These qualitative changes are clearly visible, but standardization of the description is difficult. A number of similes has been used: 'light-spot', 'light-band' 'network', 'fish-scales', 'cobweb' and 'turtle-back calcifications' (Araki *et al.*, 1985; Liu, 1985; Murakami, 1986). The changes observed appear to be specific for *S. japonicum* infection. The 'network' pattern is anatomically separate from the portal venous system. It is found more often in adults than in children, suggesting long-standing lesions (Kardorff *et al.*, 1999a). Cholestasis and enzyme changes indicate liver damage in these cases (R. Kardorff and R.M. Olveda, personal communications). 'Network' patterns of the liver texture were also described in Wilson's disease (Kaneko *et al.*, 1989), but the nodules are smaller than those usually seen in *S. japonicum* infections. Structural changes of the liver due to chronic hepatitis B may also resemble schistosomal lesions, as is also seen in *S. mansoni* infections (Zheng, 1989). The spaces, however, are usually smaller in hepatitis pathology, giving the impression of a tangled 'meshwork' rather than a 'network'. Subcapsular and septal calcifications in peripheral (and central) locations, which have been shown to harbour *S. japonicum* eggs, produce a characteristic 'turtle-back' appearance (Araki *et al.*, 1985).

The prevalence of hepatomegaly peaked in the fourth decade of life among 825 subjects examined in China, and that of periportal thickening did so in the fifth decade (Wiest *et al.*, 1993). A progressive increase in the extent of periportal thickening with age was observed, together with a higher prevalence among men than among women despite a similar prevalence and intensity of infection with *S. japonicum* in both sexes.

3.4.2. *Evolution of Lesions*

Regression of clinical symptoms, of hepatosplenomegaly and of ultrasonographic changes after treatment has been reported from different endemic areas (Chen *et al.*, 1981; Rubio, 1982; Hadidjaja *et al.*, 1985; Cai *et al.*, 1997). Ohmae *et al.* (1992b) followed 52 patients with *S. japonicum* infections during 6 months after treatment with praziquantel. Marked reduction in the thickening of the portal vein wall was recorded. However, no improvement was observed in those patients in whom enlarged collateral vessels had been demonstrated. A longitudinal cohort analysis of Chinese patients with

periportal thickening showed signficantly better regression of grade II and grade III thickening in treated than in untreated subjects (Wiest *et al.*, 1994). Both hepatic enlargement and fibrosis, but not the 'network' pattern, appear to be reversible with aggressive treatment (Olds *et al.*, 1996, Cai *et al.*, 1997; Lin *et al.*, 1997).

3.4.3. *Key Issues of Morbidity due to* S. japonicum

Early development of morbidity is a striking feature of *S. japonicum* infection. It is related to the high daily egg output. The lesions detected by ultrasound correspond to those seen in *S. mansoni* infections, with the exception of a 'network' pattern in the liver parenchyma, an anatomical structure which is not related to the portal venous system (Yi and Wong, 1992). Periportal thickening is at least partially reversible, but 'network' pattern changes tend to persist, possibly because they are related to longer-standing pathology. The latter morbidity is predominantly seen among adult individuals. As with *S. mansoni* infections, repeated treatments appear to be necessary for sustained reduction of morbidity.

3.5. *Schistosoma mekongi*

S. mekongi infection was first detected in the late 1950s in a patient from Laos at a hospital in Paris. The first investigation of morbidity in an endemic focus using visualizing methods was carried out in 1996 by Hatz *et al.* (1996), who described the ultrasonographically detectable morbidity due to *S. mekongi* in a focus in the north-eastern province of Stung Treng in Cambodia; 299 individuals from Sdau, a village on one of the tributaries of the Mekong, were investigated. The parasitological prevalence of *S. mekongi* was 73% (163/223), based on stool samples (Kato–Katz thick smears). Pathological changes were found in 84% of all subjects (mean age: 19 years; range 3–69 years); 16% of the subjects had substantial periportal thickening. Few cases of severe pathological lesions were recorded. No case with ascites was seen. A few cases of gastrointestinal bleeding were reported anamnestically.

The prevalence of pathology was similar in all age groups: 3–14 years, 81%; 15–20 years, 86%; 20–69 years, 87%. Children less than 15 years of age were significantly more often infected and had significantly more heavy infections (>400 eggs per gram of stool) than older individuals. Periportal thickening was detected more often among the younger than among the older age groups ($P<0.05$). Portal vein enlargement was found more often among older subjects, but this difference was not significant. Spleen size measurements were inconclusive as malaria was also prevalent and could have

contributed to splenomegaly. A survey conducted among the village population a few weeks earlier had shown a prevalence of 33% for malarial parasites. However, in only 3% of the study population were malaria parasites detected at the time of the ultrasound survey.

The pathological lesions observed were similar to those reported in infections with *S. mansoni* rather than with *S. japonicum*, with the exception that parenchymal hyperechoic lesions were found in 9% of all cases. These lesions, which are not attributed to the portal vein system, have previously been reported in *S. japonicum* infections only. It is possible that very little other pathology resembling *S. japonicum* lesions was found in this setting because the focus was, apparently, relatively recent, as reported by the villagers, so the severity of chronic lesions was less marked than in areas highly endemic for *S. mekongi* (see Biays *et al.*, 1999).

The study was the first to evaluate both descriptive and quantitative methods to assess liver pathology (WHO, in press). Whereas the former assesses the appearance of liver texture according to a pretested image pattern, the latter measures the thickness of the walls of the first-order segmental portal vein branches. The results reported in this study favoured the image pattern method in this setting, as rigorous application of the measurement method would have missed up to 10% of livers with pathological changes.

No published report from other endemic areas, or of follow-up investigations after treatment, exists. High reinfection rates following treatment have been reported in other Cambodian foci along the Mekong river (Biays *et al.*, 1999; Stich *et al.*, 1999). A follow-up study using ultrasound is currently being carried out among a cohort of 93 children in Stung Treng province.

4. THE APPLICATION OF ULTRASONOGRAPHY IN SCHISTOSOMIASIS

4.1. The Acceptability of Ultrasound Examination: a Question for Inter-disciplinary Research

Ultrasound is an established tool for investigating pathology due to schistosomiasis in the field. Little has been reported on such investigations outside hospital settings with regard to the acceptability of the method, especially with regard to religious and gender aspects. Acceptability cannot be taken for granted, since the ultrasound examination is carried out in a dark room by an unknown doctor and the probe is placed on several parts of the abdomen, including the critical area above the pubic symphysis.

The acceptance of ultrasound examination was investigated in two rural communities in south-eastern Tanzania, among 531 schoolchildren (mean age,

12.4 years, range 7–18 years; male/female ratio 0.9) undergoing a follow-up study on *S. haematobium* pathology (Hatz *et al.*, 1998). The children were divided into younger (7–12 years) and older (13–18 years) groups; 67% of the subjects stated they were Christians and 33% Muslims. The examining team consisted of two African doctors (male) and two Europeans (one female, one male). Boys and girls were examined separately and a teacher of the same sex assisted during the examination. Children were interviewed in private immediately after the ultrasound examination by two female teachers (59% of interviews) and one senior male field worker (41% of interviews) in the local vernacular. The interviewers were selected because of their particularly good and easy-going rapport with the children. The interviews were supervised, out of earshot, by one of the investigators. The structured interview included five questions regarding pain (*kusikia maumivu*), fear (*kuogopa*), feeling of shame or embarrassment (*kuona aibu*), and preference regarding sex and origin of examining doctor (African or European). The question about pain, with an expected high frequency of negative answers, was included to test whether the interviewers were getting appropriate answers. Ultrasound scanning is not painful unless the bladder is very full.

The interviews were conducted under field work conditions. The constraints of time, infrastructure, and the convenience of the pupils and helpers did not allow for paired analyses or for matching; 531 children (285 girls and 246 boys) answered the first four questions and 481 responded to all five. The last question was included only on the second day of the nine days' survey.

As expected, 88% of the subjects denied feeling any pain during the examination; the positive answers were given by children who stated that they had a very full bladder. Nearly all (93%) of the respondents were not afraid of the examination, but 6% reported a slight, and 1% (n=7) a strong, feeling of fear. Explanations given for fear included 'it may hurt', 'the device may cut the skin', and 'it [the ultrasound probe] is cold'. Girls admitted significantly more often to having been afraid (P= 0.001). Age had no influence on this issue.

The question regarding the experience of shame during examination was negatively answered by 94% of the subjects, 4% acknowledging a slight, and 2% a strong, feeling of shame. Reasons for shame included 'because there are many eyes in the room' and 'the probe is placed near the private parts'. Girls felt shame or embarrassment significantly more often than boys (P=0.001), but no difference was found between the two age groups of either sex. The sex of the examiner was relevant, girls admitting feeling embarassed more often when examined by a male doctor. However, individual characteristics of behaviour by the examiners seemed to be important, as each of the three male doctors provoked significantly different answers from the pupils.

The religion of the child was relevant: significantly more Christian than Muslim respondents acknowledged feeling embarrassed during the examination. This held true for Christian versus Muslim girls as well.

Asked whether they would prefer a male or female examiner, 38% of the girls and 48% of the boys chose a doctor of their own sex ($P<0.05$). Among the girls, 54% stated they had no preference in this respect (significantly more young girls than older ones stated a preference for a female examiner), whereas the figure for the boys was 47% (not significant). Few children would have chosen a doctor of the opposite sex. The religious factor was again relevant: Muslim children showed a greater tolerance regarding the sex of the examiner. 50% of 481 children stated no preference when asked if they preferred to be examined by an African or European doctor. Among Muslim children the proportion was 60%. Among those who stated a preference, 29% of all children would have preferred an African doctor and 21% a European one. Reasons given for the preference included 'he/she has the same colour of skin' for African preference and 'he/she is an expert' for the European preference. Children stating no preference said that 'it does not matter who examines me because they are both medical doctors and should know their business'.

The results showed that ultrasound examination is well accepted in the setting described. The method is to some extent gender-sensitive. Girls more often acknowledged a feeling of embarrassment or shame during the examination than male pupils. Nevertheless, a considerable percentage of the boys, if given the choice, would also prefer to be examined by a doctor of their own sex.

Surprisingly, the results showed a greater tolerance by the Muslim children regarding the gender issue and the ethnic origin of the examining doctor. From the socio-cultural perspective, these results are bound to question general ideas about the influence of the Muslim faith on attitudes and behaviour. Our findings are not in line with the information compiled by Feldmeier and colleagues (1993), who suggest a greater gender-sensitivity of Muslim patients. Age is certainly an important factor; adult women might have reacted differently. However, I had an interesting experience during a survey examining the entire population of one rather closed Muslim community on Pemba Island (Tanzania). The participation of women of all ages in ultrasound examination, which was carried out under favourable conditions (privacy), was enthusiastic, although it was performed by a male examiner. A female health care worker was always present during the ultrasound investigations. The women knew from previous surveys that ultrasound is also used to detect pregnancy, and the positive connotation of the ultrasound examination, a possibility of gaining an insight into their reproductive system, outweighed by far any feeling of embarrassment during the examination.

In conclusion, abdominal ultrasound examination in a darkened room appears to be acceptable among children and adolescents, but the training of female as well as male medical staff should be promoted, so that everyone has the option of being examined by a doctor of the same sex. Circumspect and informed handling of the patient, the presence of a relative or other close person, and privacy are eminently important during this sensitive examination.

Social categories as complex as religion are best used in a discriminating and informed manner, based on anthropological and ethnographical information about the region and group concerned. The same applies to labels like 'ethnic' or 'cultural and/or behavioural peculiarities' (Feldmeier *et al.*, 1993). These categories and identities are often constructed and applied by outsiders and seldom represent the reality of the people concerned.

4.2. Optimizing the Tool: from Cairo 1990 to Niamey 1996

A group of scientists, clinicians and control officers with wide experience of the application of ultrasound met under the auspices of WHO/TDR in Cairo, Egypt, in 1990 to discuss the use of ultrasound in schistosomiasis. The aims of this meeting were (i) the standardization of findings detected by ultrasound and (ii) recommendations for the use of ultrasound in schistosomiasis control programmes. The group made tentative proposals (WHO, 1991) for a set of standard investigations to be evaluated in the field. They were designed to obtain essential information on the key pathology in a large number of individuals under field conditions, allowing only about 5 minutes to examine each person, and using portable equipment. The following criteria were considered by the participants of the Cairo workshop to indicate the most important investigations.

1. Lesions which indicate that the disease is likely to develop into a severe form. The following lesions may predict a severe outcome: (i) moderate stages of periportal thickening as an indicator of fibrosis, leading to portal hypertension and oesophageal varices (bleeding risk) in *S. mansoni* and *S. japonicum* infections; (ii) early stages of hydronephrosis in *S. haematobium* infections, leading to a non-functional kidney.

2. Lesions that are considered to be typical of chronic infections. In certain endemic areas (e.g. Egypt), bladder wall calcification is pathognomonic of *S. haematobium* infection; in other areas (e.g. East Africa) it is seen less often. Extensive disturbances of the liver texture – Symmers's 'pipe-stem' fibrosis in *S. mansoni* and the 'network' pattern in *S. japonicum* infections – may be found in patients with advanced disease.

3. Ultrasound examinations must be selected that provide simple and unambiguous images that can be identified reliably and measured accurately.

4. Lesions which are likely to change in response to treatment. This is important for the evaluation and monitoring of treatment and control programmes.

5. Investigations with a high level of specificity. Those which are likely to produce many false-positive results should be avoided. A high level of specificity is more important than optimal sensitivity. One of the aims of collecting data on pathological lesions is to assess the efficacy of interventions in reducing the frequency and the level of such pathology in a population. Efficacy

measurements are based on the estimation of risk, e.g. the risk of lesions developing or the chance of regression. The relative risk decreases if there are large numbers of false-positive results (lower specificity), whereas it is not affected by false-negative results (lower sensitivity).

The publication of these tentative proposals for standard investigations produced a wide range of reactions from investigators who used them in the field. Some investigators adopted the proposed standards for their studies and some refined them by careful and critical appraisal. New studies also investigated issues that had not been tackled before the Cairo meeting. Thus, the mimeographed report (WHO, 1991) and the subsequent publications (Hatz *et al.*, 1992a, b, c, d) created the momentum which was intended by the organizers of the Cairo meeting.

After a substantial amount of work had been done, and it had become evident that some of the standard recordings for pathology due to *S. mansoni* needed revision, a second consensus workshop was convened by WHO in Niamey in 1996. The aims of this meeting were to fill the gaps in knowledge about morbidity patterns due to *S. mansoni* and *S. haematobium* as revealed by ultrasound examination, and to review the possibilities of the use of ultrasound in control programmes. Issues pertaining to *S. japonicum* could not be addressed in detail, as many of the researchers working on this type of schistosomiasis could not attend. Few data on ultrasound investigations from endemic areas in China and the Philippines have been published in accessible literature since 1990. A further meeting to discuss the Asian forms of schistosomiasis is planned by WHO when further data become available.

The participants in the second workshop reviewed work done since 1990, and discussed the recommendations made for the most important investigations in Cairo in the light of new evidence (WHO, in press).

1. Risk of bleeding from oesophageal varices due to *S. mansoni* infection. Several indicators of hepatosplenic pathologies were found to be associated with oesophageal bleeding. Scores for this association were proposed. One of them was positively tested in a prospective study (Richter *et al.*, 1998: see Section 3.2, p. 244).

2. Risk of renal impairment in patients with *S. haematobium* infection. No new information on the impact of hydronephrotic changes due to *S. haematobium* on renal function and mortality has been published since 1990. Long-term studies using methods to measure renal function over time would be welcome, but may be ethically unacceptable. A properly designed case–control study in an endemic area where good diagnostic tools are accessible may answer some of the open questions.

3. Pathognomonic lesions and geographical differences. Decisions on which lesions due to *S. mansoni* and *S. haematobium* are pathognomonic were confirmed in further studies. Recent reports on the pattern and extent of pathological lesions due to *S. mansoni* indicated that there are differences between

West African and other African populations which cannot be attributed solely to differences in intensity of infection and exposure. Further investigations are required to understand the differences. Similar variation may also occur in *S. japonicum* infections in China and the Philippines.

4. Unambiguous images of morbidity. Experience has shown that lesions which appear early in the development of *S. haematobium* and *S. mansoni* morbidity are subject to inter-observer variance. This should encourage the quest for simplified methods. The protocols as modified in Niamey should reduce the inter-observer bias and thus produce a more clear-cut set of information for comparing patterns of lesions from different settings. This advantage, however, is overshadowed somewhat by the fact that, when only clear-cut lesions are considered, pathological early lesions and those which have regressed as a result of treatment may be missed. These include the lesions most likely to regress after treatment (periportal thickening in *S. mansoni* infections and bladder lesions with *S. haematobium*) (Doehring-Schwerdtfeger *et al.*, 1992a; Hatz *et al.*, 1998).

5. Observation of changes in response to treatment. Almost all lesions due to *S. haematobium* resolve in children and adolescents within 1 year. The exact speed of resolution has not yet been determined as the intervals between the surveys in the published reports were too wide. The resolution time for obstructive lesions of the upper urinary tract has still not been agreed upon by various investigators and there may be geographical differences (Hatz *et al.*, 1990a, 1998; Wagatsuma *et al.*, 1999; G. Campagne, personal communication).

Periportal thickening was shown to regress significantly, but not to disappear, within 23 months after treatment. The rate of hepatomegaly decreased from 11% to 7%, but the rate of splenomegaly increased during the period of observation (Doehring-Schwerdtfeger *et al.*, 1992a).

6. Specificity. The number of false positive results reported will depend largely on the threshold selected to distinguish 'pathological' from 'normal'. If it is too low, there will be too many false positives, and vice versa. The new protocols prepared in Niamey take this into account. Ideally, the cut-off point for any lesion should be validated against an independent 'gold standard'. However, for early lesions in schistosomiasis such standards are hard to define and even harder to apply under the study conditions where they are most needed. For example, in the case of liver lesions, examination of liver biopsies could provide a standard, but this procedure is not ethically justifiable in early stages of the disease, and moreover it would not be reliable owing to the focal distribution of the lesions at this stage. Connective tissue metabolites have so far not been shown to be useful as diagnostic serum markers (Kardorff *et al.*, 1999b). In the future, novel fibrosis markers measured in the serum or the urine, and magnetic resonance imaging, may offer a standard, but further validation is needed. Because of the problem of defining cut-off points, the term 'borderline' pathology has been integrated into the standard protocols.

The main conclusions from the Niamey meeting can be summarized as follows.

1. Standardization of pathological lesions due to *S. haematobium* appears to be reliable. The following minor changes to the protocols were made to render the ultrasound investigation less ambiguous and more easily applicable in the field.

(a) Fissures of the renal pelvis (up to 1 cm width) are now considered to be within the normal range even though they may reflect an early stage of urinary tract obstruction (Hatz *et al.*, 1990b, 1992a). This decision was made to minimize further the inter-observer variation in this finding, which is not considered an indicator of important pathology.

(b) The special aspects of investigating pregnant women need to be considered: satisfactory filling of the urinary bladder is often not possible due to mechanical compression; dilatation of the ureters and the pyelon may be present in the absence of schistosomal pathology due to hormonal factors or compression. These circumstances can lead to interpretational errors in the examination (Richter *et al.*, 1996).

(c) New scores for assessing pathological lesions due to *S. haematobium* were proposed, whereby lesions of the upper urinary tract are given more weight than bladder lesions (Medhat *et al.*, 1997; Hatz *et al.*, 1998; WHO, in press).

2. All quantitative recordings of the liver and spleen have to be adjusted for the patient's height. These measurements may also be influenced by the observer's subjective interpretation of the image. As they are also time-consuming, they are not ideal for field use.

3. The classification of pathological lesions due to *S. mansoni* proposed in Cairo was shown by studies in different geographical settings to be inadequate in practice. The cut-off values suggested for measurements of hepatic pathology tended to overestimate early stages of hepatic involvement (periportal thickening, portal vein diameter). The dependence of periportal thickening, portal vein diameter and other liver and spleen measures on body height were not considered in the Cairo classification. The importance of this variation was shown by various investigators from different endemic settings (Kardorff *et al.* 1996; Yazdanpanah *et al.*, 1997; J. Richter, unpublished data). The distinction between normal and mildly abnormal findings remains a point of discussion. It was estimated that minor periportal thickening may be overestimated by more than 50% (82% vs. 30%), but higher grades of periportal thickening would be correctly assessed (9% vs. 10%) in community studies. A revised, quantitative method, measuring two first-order segmental branches of the portal vein tree, was agreed upon. A measurable standard for follow-up investigations and comparison of data between different endemic settings is facilitated by a quantitative approach. However, practical experience with the Cairo classification over 6 years indicated that there is considerable debate

among investigators on the practicality of such measurements. Pathology tends to be focally distributed in the liver. If no pathology is found on the branches indicated in this report, the risk of false negative results is high because lesions on other parts of the portal tree may not be recorded. A descriptive method, as outlined in previous studies (Homeida *et al.*, 1988b; Doehring-Schwerdtfeger *et al.*, 1989) and further developed and field-tested by J. Richter, was accepted as an alternative. It was thus decided to include both methods in the present protocol with the results recorded as separate scores. Future application of the two recordings will show whether they are complementary to each other or whether one is superior. Most participants in the Niamey workshop agreed to evaluate the two recording methods and to convene again to discuss them. It remains clear that both assessments lack proper validation by 'gold standard' measures such as histopathological evidence, or even by other imaging techniques. MRI appears to be an appropriate technique to assess and monitor schistosomal pathology of the liver (Patel *et al.*, 1993). Unfortunately, it is clearly impractical in the field and hardly available in many endemic areas even in diagnostic centres.

4. The finding that hepatitis C infection is highly associated with schistosomiasis in Egypt merits more ultrasound investigation to further the understanding of liver lesions in such co-infections (Angelico *et al.*, 1997).

5. CHEMOTHERAPY AND ULTRASOUND IN SCHISTOSOMIASIS CONTROL PROGRAMMES

5.1. Present Status of Measures for Schistosomiasis Control

The most important and effective tool for schistosomiasis control at present is drug treatment with praziquantel (WHO, 1999). The cost of praziquantel (US$0.30–0.40 per patient) and distribution constraints are currently the most important obstacles to its use. Therefore, it is important that treatment with this drug should be carefully targeted to produce the maximum effect. Decisions about where, for whom, and how frequently it should be used must be based on the rapid assessment and monitoring of schistosomiasis endemicity in old and new foci, on knowledge about the most vulnerable groups, and on an understanding of the way in which morbidity develops over time and responds to treatment.

Some countries, such as Brazil, China, Egypt and Morocco, have managed to install and successfully run control programmes and some have even achieved the eradication of schistosomiasis as a public health threat. Other areas, including most sub-Saharan African countries, where only six national or regional control programmes exist, are lagging behind. Among the measures

which have proved most effective are repeated treatments of people in known highly endemic areas and targeting of schoolchildren. This age group is most affected, most likely to show clinical disease and pathological lesions and, consequently, most responsible for reinfecting a community.

Since a vaccine to prevent infection and morbidity is not yet available, health education, water supply, sanitation and snail control are the only current methods available for schistosomiasis control. Oxamniquine and metrifonate are the known alternative drugs for treating cases of schistosomiasis but, in contrast to praziquantel, they are active against only some species. Moreover, they are in danger of disappearing from the market because of financial considerations. Therefore, only one effective drug to treat all schistosomiasis cases is currently available world-wide. Even praziquantel, of which several brands exist, is not uniform in its effectiveness (Guyatt and Chan, 1998). There is no hard evidence for drug resistance to praziquantel among any of the schistosome species yet, but the risk of such a development remains a threat and the efficacy of praziquantel must be closely monitored (Cioli, 1998). The development of new alternative drugs is thus urgently needed.

One novel approach is the use of artemisinin derivatives for treatment of early schistosomal infection (Xiao *et al.*, 1996; Utzinger *et al.*, 2000). Artemether has been shown to kill cercariae and schistosomulae and thus prevent the development of adult, egg-laying worm pairs. It has been tested in animals and in seven double-blind, placebo-controlled trials involving more than 4500 individuals exposed to *S. japonicum* infection (Xiao *et al.*, 2000). Laboratory experiments indicated activity against *S. mansoni* in mice (Xiao and Catto, 1989). Efficacy against *S. mansoni*, *S. mekongi* and *S. haematobium* in humans has not yet been investigated. The drug may be used prophylactically among well-defined high risk groups such as fishermen and children in endemic areas, and among travellers exposed to accidental exposure. In areas where malaria is endemic, any use of the drug against schistosomiasis must be assessed against the possibility of promoting artemether-resistant malaria parasites.

5.2. The Contribution of Ultrasonography

Ultrasound studies have contributed to the understanding of schistosomal pathology by providing objective data on lesions and their evolution over time. Since ultrasound was introduced as a diagnostic tool in community-based surveys of *S. haematobium*-related pathology (Degrémont *et al.*, 1985; Doehring *et al.*, 1985a), its application for screening populations in control programmes has rapidly spread to many endemic countries with all three major schistosome species (Heurtier *et al.*, 1986; Lamothe *et al.*, 1988, 1989;

Doehring-Schwerdtfeger *et al.*, 1989; Ohmae *et al.*, 1992a). Cross-sectional and longitudinal surveys among populations in endemic areas have provided important information on schistosome-related pathology for public health decisions, for example how to determine the most appropriate time interval for treatment of populations in endemic areas. This question has been addressed by various investigators with the help of ultrasound studies (Homeida *et al.*, 1996; Boisier *et al.*, 1998; Hatz *et al.*, 1998; Wagatsuma *et al.*, 1999).

In a study conducted from 1988 to 1994 in Sudan, Homeida and colleagues (1996) found that repeated chemotherapy was necessary in areas of high transmission of *S. mansoni*. Two rounds of annual mass chemotherapy significantly reduced morbidity and infection, whereas biennial chemotherapy only reduced the intensity of infection. In high transmission areas such as the newly detected focus in Senegal (see Section 3.2.3, p. 250), it is necessary to conduct repeated mass treatments at short intervals in order to control the development of morbidity (Picquet *et al.*, 1998).

A study by Doehring-Schwerdtfeger and colleagues (1992a) indicated that chemotherapy can improve pathological lesions in the presence of reinfection, though the study did not specify whether the infections were due to persistence of some of the parasites or to reinfection. It also suggested that, in that endemic area, re-treatment after successful initial treatment may not be necessary until two years have elapsed. Regression of morbidity one year after treatment with oxamniquine was reported from an endemic area in Brazil (Santos and Coura, 1986). This was followed by an increase of morbidity 5 years later, indicating that re-treatment would have been appropriate after about 3 years. Boisier and colleagues (1998) suggested two rounds of annual chemotherapy given to the entire population, followed by annual targeted mass treatment for children and adolescents, in areas with initial high prevalence. As in *S. haematobium* endemic areas, the optimal treatment intervals to prevent substantial resurgence of pathology may need to be defined for various settings with different endemicity (Hatz *et al.*, 1998). For *S. mansoni* infection in moderate to high transmission areas, two treatments 40 days apart (Cioli, 1998) and re-treatment after three or more years appear to be a reasonable compromise, based on existing reports (Doehring-Schwerdtfeger *et al.*, 1992a). However, more information must be gathered from longitudinal studies in different endemic settings in order to validate such recommendations, and close monitoring of such efforts are pivotal in areas where no previous study has been conducted.

With *S. haematobium* infections, it was found that chemotherapy with a standard dose of 40 mg/kg of praziquantel was adequate in most endemic areas to reduce morbidity successfully. There appears to be no need for mass treatment more than once yearly (WHO, in press). The level of endemicity governs the timing of re-treatment. In moderate to high endemicity areas with rapid reinfection, one mass or target group treatment every 2 years may be

appropriate to prevent morbidity of individual and public health importance (Hatz *et al.*, 1998). In areas with low transmission and reinfection rates, the interval may be 3 to 4 years (Wagatsuma *et al.*, 1999). Information from other areas is needed for decision making. The outcome of repeated chemotherapy can be monitored by recording the evolution of pathological lesions in relation to parasitological treatment failures, reinfection and insensitivity of screening. Models for morbidity, including measures of direct and indirect morbidity that distinguish mild, possibly spontaneously resolving lesions and morbidity predicting severe complications in individuals, can help to optimize the treatment intervals necessary in different endemic settings.

Apart from longitudinal and well-designed cross-sectional studies on all major *Schistosoma* species, papers on specific questions such as the identification of patients in whom the infection may lead to bleeding from oesophageal varices have been published (Homeida *et al.*, 1988b; Richter *et al.*, 1998). Whether such models could also allow an estimation of the probability that an individual would seek treatment for early symptomatic disease (Gryseels, 1996), leading to improved passive case detection, remains to be seen. In addition, cost-effectiveness modelling could further contribute to a rational selection of control strategies (Guyatt and Tanner, 1996).

5.3. Identification of Target Groups

Treatment campaigns have often concentrated on schoolchildren. There are several reasons for this. They represent the age group with the highest prevalence of the disease in most endemic settings, compliance is high, and follow-up is relatively easy because access is provided through the educational services. Studies using ultrasound have shown that, in addition, reaching children between the ages of 5 and 15 years is important in terms of reducing transmission and curtailing the effects of infection before permanent damage is done to the organs.

For studies using ultrasound, there is the additional advantage that – as experienced in all studies so far – schoolchildren are not afraid of this unusual examination.

5.4. Relationship of Ultrasound Observations to Other Measures

Even though ultrasonography is becoming more widely available, the bulk of information on schistosomiasis is likely to consist of clinical and laboratory data. It remains to be established to what extent such findings reflect the actual morbid anatomy of the disease and its functional consequences. In areas with various levels of endemicity, applying the exploratory case–control technique

can be an appropriate basis for surveys to clarify this question. Cases are defined on the basis of morbidity classes, and appropriate controls are selected. The association of lesions with egg counts or other indirect morbidity indicators can then be assessed. The strength of the association can be calculated and the relative risk estimated. This approach helps to identify major relationships and new findings which may then be investigated by in-depth studies with appropriate sample sizes.

Ultrasound findings have been shown to correlate well with egg output and other indirect measures of schistosomal morbidity among populations in endemic areas (Murakami, 1986; King *et al.*, 1988; Abdel-Wahab *et al.*, 1989; Hatz *et al.*, 1990b; Kahama *et al.*, 1998). However, the correlation between ultrasound findings and laboratory tests is not always consistent. This variability may be due to various factors (Hatz *et al.*, 1990b). One is the question of methodology: for example, egg output varies diurnally and from day to day, so that if urine and stool examinations are done only once or twice, a person with detectable pathology may not be shedding eggs at the time of examination. Another possibility is that pathology is present but eggs are not being excreted: the eggs may remain trapped in lesions, especially in long-standing infections, or pathology may still be present although the infection is no longer active. In some areas, some of the observed pathology may be due to diseases other than schistosomiasis. An accurate knowledge of the disease pattern in an area is essential before the connection between ultrasound observations and schistosomal infection can be accurately assessed.

Associations between pathological lesions and other measures of infection have been widely documented. However, there are only a few estimates of the extent to which pathological lesions associated with schistosomiasis can be attributed to infection as determined by egg output or microhaematuria measurements (Hatz *et al.*, 1998). Attributable fraction (AF) estimates have been shown to be useful in previous work on geohelminth and schistosomiasis infections (Guyatt *et al.*, 1995; Booth *et al.*, 1996; Booth, 1998). AF estimates were calculated at baseline in a longitudinal study in Tanzania. As expected, AFs were higher in those with severe pathology than in the combined pathology classes (Hatz *et al.*, 1998). Combined infection markers (egg count and/or microhaematuria) gave higher AFs for major pathology than any of the two markers alone, but egg counts alone gave higher AFs for overall bladder lesions. The high prevalence of bladder lesions was considered to be a possible explanation of this puzzling finding, though the possibility of lesions unrelated to schistosomiasis cannot be eliminated.

In the same study, the positive predictive potential of two different levels of egg counts for pathology among children with no lesion at baseline and among those with reappearance of lesions after clearance was demonstrated. Pretreatment figures were similar to those at 24 months, indicating that this measure could be used in the absence of ultrasound data as an indirect marker

of pathology. The predictive potential at 6 to 18 months was considerably lower because treatment affected the relationship between egg output and pathological lesions of the urinary tract at early stages of the reinfection cycle.

5.5. Monitoring During Control Programmes

When control programmes are implemented, the pattern of morbidity can be expected to change. The relationship between ultrasound, egg counts and indirect morbidity indicators needs to be established throughout control programmes in settings of various reinfection patterns. These relationships have so far rarely been evaluated throughout a control programme, from the attack phase to the maintenance phase, with regard to their predictive potential in situations where there is changing prevalence of morbidity. Some studies of this kind have been carried out for microhaematuria, circulating worm and soluble egg antigens, and eosinophilic cationic protein measurements in urine. These measures proved to be useful tools for surveillance during a control programme (Kremsner *et al.*, 1994; Lwambo *et al.*, 1997; Kahama *et al.*, 1998, 1999; Reimert *et al.*, 2000).

Ultrasonography can be used to assess subsamples of the population covered by a control programme, and can thus confirm and validate the impression gained by indirect methods about sex- and age-specific patterns of pathology (Hatz *et al.*, 1992d). Monitoring can continue during the maintenance phase, and decisions must be made about an appropriate sampling strategy, using clusters and possibly morbidity cohorts, to be followed. These decisions will have to be made on the basis of the original cross-sectional survey.

5.6. The Future Role of Ultrasonography

Ultrasonography is playing an increasingly important role in studies of schistosomiasis morbidity. To strengthen its role further, more investigations are needed in different areas into the course of development of morbidity patterns and the dynamics of changes in lesions due to schistosomiasis (especially hepatic fibrosis) after single or repeated treatments, in order to adapt treatment strategies to regional particularities. Geographical variations need to be further clarified. New endemic foci as well as epidemic outbreaks of schistosomiasis need to be studied and monitored.

There is a need to establish regional and height-related standards for organometric values in healthy subjects. Identification of individuals in whom pathology does not regress, or only partially regresses, after treatment is important for focusing efforts to understand the possible impact of incomplete

eradication of the worm load in individual patients after single or repeated treatment with drugs. The investigation of immunological and possibly genetic mechanisms is also important in this context.

New markers of morbidity in relation to ultrasonographically detected lesions should be identified and validated and, in addition, the relationship between pathological lesions and perceived morbidity should be explored.

In individuals infected with *S. haematobium*, the impact of schistosomal urinary tract pathology on renal function and on mortality needs to be evaluated. In *S. mansoni* infections, the documentation of the dynamics of portal hypertension and the critical assessment of treatment effects, using Doppler and conventional ultrasound, are research topics of importance. The pattern of pathological lesions due to *S. intercalatum* awaits exploration. In infections due to *S. japonicum* and *S. mekongi*, the natural history of lesions detected by ultrasound needs to be understood, and standardization of pathological lesions should be established. The relationship between lesions due to schistosomiasis and concomitant infections (e.g. viral hepatitis) or non-infectious diseases (e.g. liver cancer) needs to be evaluated.

In-depth investigations of schistosome-related morbidity of the reproductive system deserve special attention. The importance of lesions in the genital tracts of female and male patients needs to be investigated with regard to resolution after treatment and to their role as potential causes of infertility, entry points for other infectious diseases, and neoplasms. Furthermore, the influence of schistosomiasis on fetal growth needs to be studied further.

Ultrasound can be used in surveys and control programmes at community level, though it remains a technique for specialized teams, to be performed on subsamples of a population. It is also invaluable in hospitals for the better diagnosis and treatment of severe cases. Its use for the assessment of early hepatic, genital and urinary tract lesions or of bladder calcification needs further refinement. However, ultrasound is already an invaluable tool to complement and validate the indirect morbidity control measures that can be assessed by the existing health care services in many areas (Tanner, 1989a, b). In this context, it forms an essential basis for schistosomiasis control programmes.

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Ascaris and ascariasis

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ABSTRACT

In recent years much new information has been obtained about the epidemiology, population biology and public health significance of infections of *Ascaris lumbricoides* in humans. Results from experimental infections of *A. suum* in pigs have helped to elucidate the observations made in the community on human ascariasis. The main purpose of the review is to see how new information may contribute to further acceptance of ascariasis as a serious contributor to ill-health and so to the design and implementation of sustainable control programmes intended to reduce the morbidity due to infection with *A. lumbricoides*. Eradication is neither a realistic nor prudent aim given the current shortage of appropriate sanitation in many countries where ascariasis is endemic. A substantial body of evidence shows that for the four common species of soil-transmitted nematode, including *A. lumbricoides*, regular administration of broad-spectrum anthelminthic drugs to children attending primary schools is a cost-effective means of controlling the infections. Anthelminthic drugs must be of proven quality and efficacy and health professionals should be prepared to detect and manage drug resistance should that emerge. Despite a deeper understanding of the immune response of a variety of hosts to infections with either *A. lumbricoides* or *A. suum* there is at present little prospect of an effective vaccine against ascariasis. The relationship between *A. lumbricoides* and *A. suum* is addressed, particularly since both species, if they are indeed separate species, occur in people and their pigs in many communities.

1. INTRODUCTION

Although the organism we now call *Ascaris lumbricoides* has been known to its human hosts for many hundreds of years (Grove, 1990; Goodwin, 1996), it was not until the late 17th century that the results of scientific investigations were published by Tyson (1683), in which he proved conclusively that the worms he retrieved from people were not earthworms because they had separate sexes with distinct gonads and reproductive organs. Tyson appears to have

questioned the prevailing idea that intestinal worms arose by spontaneous generation; he recognized the eggs of *Ascaris* for what they are and concluded that the worms were oviparous. He wrote 'The case is plain how they propagate themselves. And Menjotius, and all before him, that were of that opinion, are mistaken; who say that these worms do not generate'.

Tyson referred to *Ascaris* as *Lumbricus teres* but Linnaeus (1758) proposed that it be known as *Ascaris lumbricoides*. Under entry 247 he writes 'corpus teres, filiforme, continuum, utraque extremitate attenuatum . . . habitat in intestinis humanis.' The validity of Linnaeus's description has remained unchallenged. Around the same time, Goeze (1782) published his description of the *Ascaris* which he called *A. suum*. It would probably have been interesting to know whether Goeze, who would have been aware of *Systema Naturae* by Linnaeus, considered whether *A. lumbricoides* and *A. suum* were the same or different species. He worked during a time when the concept of spontaneous generation was dominant and, despite Tyson's work, evidence for independent processes of infection was not sought and did not exist.

Grove (1990) provides a fascinating account of how the finding of *Ascaris* eggs in faecal samples led to an understanding of the infection process. Apparently Church (1788), despite Tyson's earlier and accurate description of the eggs, indicated that an *Ascaris* infection was acquired by the worms creeping into the mouth while a person lay asleep on the ground. Davaine (1862), however, fed embryonated eggs of *A. lumbricoides* to a rat and then found larvae in the process of escaping from egg shells in the rat's gut. He clearly concluded that the transmission of *Ascaris* from host to host was direct but other workers decided that rats and other animals served as intermediate hosts. Davaine's view was correct and the microscopical detection of *Ascaris* eggs in faecal samples, in transit from one host to another, had already been introduced by Ransom (1856) as a reliable means of diagnosis of *A. lumbricoides* infections.

Ascaris spp., particularly *A. suum* which was readily obtainable from pigs, rapidly became the standard nematode for educational and research purposes. Goldschmidt (1937), a comparative physiologist in his approach to biology, was so impressed by the current knowledge of *Ascaris* that he published a book entitled *Ascaris: the Biologist's Story of Life.* He enthusiastically submitted that 'through *Ascaris* the whole amazing tale of living creatures can be told' and further wrote that the book is addressed to everyone: 'All may find here just as much information about the living world as they need in order to walk through life with open eyes and to understand their own nature'. That claim of Goldschmidt's would now be strongly challenged and few if any modern biologists would choose *Ascaris* spp. as the model system for the study of any branch of biology. Such a claim may be justified for the free-living nematode *Caenorhabditis elegans* which has become the subject of numerous

investigations into the mechanisms of development because of its remarkable amenity to laboratory culture and genetic analysis (Brenner, 1974; see Kendrew, 1994). The obvious advantage for parasitologists and nematologists of working with *Ascaris* is its large size but, since most species of nematode tend to be small (Poinar, 1983), many workers may have overlooked problems of scaling (Schmidt-Nielsen, 1972), particularly if they have assumed that knowledge of the physiology of *Ascaris* spp. can be applied directly to other species of animal parasitic nematode living in various species of host.

Apart from the public health and veterinary significance of ascarid nematodes some fundamental principles of developmental biology have been discovered as a result of studying the chromosomes of *Parascaris univalens* (= *Ascaris megalocephala* = *P. equorum*) from the horse and *A. suum* from the pig (Muller *et al.*, 1996). Theodor Boveri (1862–1915) extended an earlier observation of Van Beneden and realized that *P. equorum* with only two chromosomes per haploid cell provided ideal material for studying the fate of chromosomes during development (Baltzer, 1967). Boveri was able to demonstrate by direct observation and elegant experiments based on centrifugation that, during the early cleavage processes following fertilization, chromosomes fragment and chromatin is eliminated from all but the cell that gives rise to germ cells. This process of chromatin diminution is probably the best known case of exception to the DNA constancy rule (Muller *et al.*, 1996) and it is now accepted that the process which determines that chromatin remains intact in the germ cell line depends on cytoplasmic factors in that cell line.

Chromatin diminution of the type described for *P. equorum* also occurs in *A. suum* but there are qualitative and quantitative differences. In *P. equorum* diminution begins after the first cleavage and about 80% of the total nuclear DNA is eliminated whereas in *A. suum* diminution begins later and about 25% of the nuclear DNA is lost from the cells destined to become somatic cells. The end result is the same for these two species in that cells originating from the line with complete chromosomes become the germ cells. Chromosome diminution has been observed in other species of free-living and parasitic nematode including *Strongyloides papillosus* in which the process contributes to sex determination rather than differentiation between somatic and germ cells (Albertson *et al.*, 1979).

Interest has resurfaced in the properties and function of the haemoglobin found in the pseudocoelomic fluid of *A. lumbricoides* and *A. suum,* studied by Davenport (1949). Under the theme of the enigmatic oxygen-avid haemoglobin of *Ascaris,* Goldberg (1995) has proposed that the function of the pigment may be to capture and supply molecular oxygen for the synthesis of sterols. Sterol synthesis involves the conversion of squalene to squalene-2, 3-epoxide in a reaction requiring molecular oxygen. Goldberg states that *Ascaris* lays thousands of eggs each day loaded with sterol. There may be some confusion

in the background to this proposal. *Ascaris* eggs each contain ascaroside formed from ascaroside esters but there is no secure evidence to show that animal parasitic helminths, including *Ascaris,* contain squalene, a finding commensurate with their inability to synthesize sterols *de novo* (J. Barrett, 1981). Apparently, studies carried out since Barrett's review have reached the same conclusion: animal parasitic nematodes do not seem able to synthesize squalene. They can produce farnesol, the immediate precursor to squalene, which is then used to produce various isoprenoid hormones (Barrett, personal communication). Goldberg (1995) notes that attempts to demonstrate squalene epoxidation in *Ascaris* extracts had been unsuccessful. So it seems that another secret of *Ascaris* remains intact.

Human infection with the four common species of nematode, collectively known as soil-transmitted helminths or geohelminths, is exceedingly widespread in the modern world. Authorities now seem to accept the frequently published estimates indicating that 1.4 billion, 1.2 billion and 1 billion people are presently infected with *Ascaris lumbricoides,* hookworms *(Ancylostoma duodenale* and *Necator americanus)* and *Trichuris trichiura* respectively (Crompton, 1999). Many individuals harbour more than one species, often for most of their life-time. The infections are found in communities where poverty prevails, where there is a lack of safe drinking water, where sanitation is non-existent or in need of improvement and where more resources are required to promote health education and support health care (Table 1).

Table 1 Features of Kenya and the Ivory Coast based on statistics published by UNICEF (1998)

Indicators		Kenya	Ivory Coast
1.	U5MR (probability of dying between birth and exactly 5 years of age)	90	150
2.	Total population (millions)	28	14
3.	GNP per capita (US $ 1995)	280	660
4.	Life expectancy at birth (years)	54	51
5.	Adult literacy rate (% over 15 years able to read and write)	78	40
6.	% of under-fives with moderate and severe underweight	23	24
7.	% total population with safe drinking water	53	42
8.	% total population with adequate sanitation	77	39
9.	% adult female literacy rate (1995)	70	30
10.	% population urbanized (1996)	30	44
11.	% government expenditure allocated to health (1990–1996)	5	4
12.	Maternal mortality rate (annual deaths from pregnancy related causes per 100 000 live births – 1990)	650	810

Until relatively recently, many health professionals appeared resigned to the view that, unless effective sanitation could be established in areas of endemic soil-transmitted nematode infection, there was little point in spending resources on treatment because re-infection would be inevitable. The persistence of these infections is a tribute to their fecundity, reproductive fitness and adaptations to transmission; every infective egg of *A. lumbricoides* seems pre-adapted to exploit the socio-economic conditions and cultural practices of the host communities. A change in the approach of at least some health professionals to infection with soil-transmitted nematode infections followed research and debate to assess their public health significance (see reviews in Crompton *et al.*, 1985, 1989; Bundy and Cooper, 1989; Pawlowski *et al.*, 1991). Recognition that these infections are deleterious to health through having negative effects on growth, nutritional status, physical fitness and activity, school performance and maternal health prompted the World Health Organization to develop and promote control strategies based on the prudent use of anthelminthic drugs of proven high quality, supported by appropriate health education and realistic sanitation (WHO 1987, 1996a, b; Albonico *et al.* 1998). Crucial to the development and implementation of this policy has been a much deeper and more secure understanding of the population biology of the nematodes and the determinants of their observed epidemiology (see Bundy, 1988; Anderson and May, 1991).

The purpose of this review is to focus mainly on the current situation with regard to the control of infection with *Ascaris lumbricoides,* referred to here for convenience as ascariasis regardless of the presence or absence of detectable disease. The reader should bear in mind the following questions: is there more to be learned about ascariasis that might be applied to strengthen control measures; is control justified; is control having sustainable effects; how cost effective are control measures; can the threat of drug resistance be offset; what benefits accrue to communities where control measures are implemented; what is the long-term future for the control of ascariasis and related helminth infections?

2. DISEASE

The nature and evaluation of the disease associated with infections of *A. lumbricoides* have proved difficult to describe and quantify. Pawlowski (1982, 1987) attempted to develop guidelines for classification of the clinical expression of intestinal ascariasis; if this scheme were to be adopted and tested it might make a useful contribution to measuring the morbidity and mortality rates associated with infection. In practice, health workers have to be aware that recognition of disease directly attributable to ascariasis is often

Table 2 Signs and symptoms of disease associated with infection with *Ascaris lumbricoides* (based on Pawlowski, 1982, 1987; Stephenson, 1987)

Stage	Event	Clinical features	Outcome
Larval migration	Migration of larvae through liver and lungs	Pneumonitis, asthma, dyspnea, cough, substernal pain	Decrease food intake
Maturation, oviposition	Presence of juveniles and patent adult worms in small intestine	Abdominal pain, abdominal distension, colic, nausea, vomiting, intermittent diarrhoea, anorexia, restlessness, anal itching, enterocolitis, disordered small bowel pattern, jejunal mucosal abnormalities	Decrease food intake Increase nutrient loss Malabsorption: protein malabsorption, fat malabsorption, D-zylose and lactose malabsorption, vitamin A malabsorption
Allergic reaction	Exposure to *Ascaris* allergen at any stage of life cycle	Hypersensitivity reactions including asthma, conjunctivitis, facial oedema, urticaria, abdominal pain, heartburn, diarrhoea	Decrease food intake Increase nutrient loss
Complications	Migration or aggregation of adult *Ascaris* in intestine	Intestinal obstruction, intussusception, volvulus Invasion of bile duct (producing obstructive jaundice, gallstones, cholangitis, or liver abscesses) Acute pancreatitis, acute appendicitis, intestinal perforation, peritonitis, upper respiratory tract obstruction	Life-threatening illnesses which all decrease food intake and may increase nutrient requirements (due to fever) and nutrient losses (due to diarrhoea)

Notes

1. Clinical expression of intestinal ascariasis
   (a) **Asymptomatic** – in well-nourished children with light worm burdens *(perhaps such cases are personally unaware of infection unless worms are observed in their stools).*
   (b) **Asymptomatic/oligosymptomatic** – host shows considerable tolerance to the presence of worms *(perhaps experiences minor intestinal changes and disturbances).*
   (c) **Symptomatic** – host appears listless and underweight with some abdominal distension with worms palpable through the abdominal wall *(caregivers will be aware that their children have worms).*
   (d) **Fatal** – resulting from severe complications.
2. Individuals not currently harbouring worms may respond to allergens.
3. In a community, most individuals harbour light worm burdens.

complicated by the presence of other infections, and the nutritional and immune status of the host. A scheme setting out the signs and symptoms associated with *Ascaris*-induced disease is set out in Table 2. Thein Hlaing *et al.* (1990a) recorded the patterns of coughing and abdominal pain in children participating in an ascariasis control programme (Figure 1). It is most important to recognize that in nearly all cases the worm burden (or intensity of infection) determines the degree of morbidity which develops and that in individuals already suffering from malnutrition or some other illness, morbidity may be manifest with a lower threshold for the worm burden.

2.1. Role of Larval Stages

According to Little (1985) there is no reliable record of larval *A. lumbricoides* having invaded visceral organs other than the liver and lungs where larval residence is limited to the period required for development. The same conclusion appears to hold for larval *A. suum* in pigs. The combination of events occurring during development as the larval *Ascaris* migrate through the host's tissue, while exposed to the host's immunological surveillance and effector mechanisms, has the potential to induce pathology. Relatively little is known directly about human–larval *Ascaris* interactions but a considerable body of information has been obtained by studying experimental infections of *A. suum* in pigs.

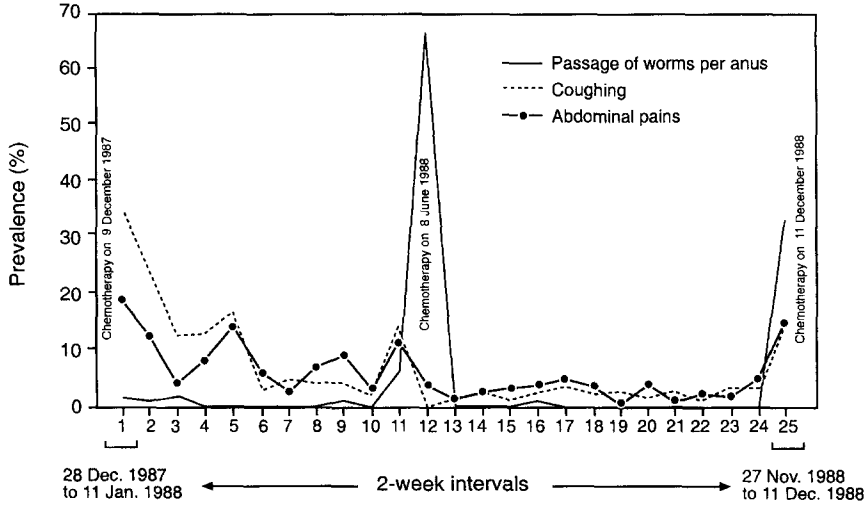

Figure 1 Results of the surveillance of various symptoms of ascariasis during a chemotherapy-based control programme. (Reproduced from Thein Hlaing *et al.*, 1990a.)

2.1.1. *Larval* Ascaris suum

'Milk spot', 'white spot' and chronic focal interstitial hepatitis are terms used to describe the lesions readily seen at the surface of livers from pigs known to have been exposed to infective eggs of *A. suum*. Similar lesions occur deeper in the hepatic tissue. Milk spots on pigs' livers are also associated occasionally with infections of *Cysticercus tenuicollis, Fasciola hepatica* and various species of nematode so their presence cannot be accepted as proof of an *A. suum* infection (Roneus, 1966). The spots have been described as representing granulation-tissue and lymphonodular-type white spots (Roneus, 1966) and localized fibrous thickening of the interlobular septa of the liver (Taffs, 1968). The longer pigs are exposed to infective eggs of *A. suum*, the greater the number of milk spots that will develop, but the number declines with continuing exposure indicating the induction of a dose-dependent host reaction (Eriksen *et al.*, 1992). Much earlier, Taffs (1968) concluded that pigs, by virtue of previous contact with *A. suum* and not as a result of age, become better able to resist larval migration after reinfection. Taffs also noted that at the same time as protective immunity is operating considerable hepatic necrosis and hepatic haemorrhage occur in pigs. Histological studies indicated that the livers of resistant pigs played an important part in the destruction of invading larval *A. suum* and in stopping their migration (Taffs, 1968). Roepstorff *et al.* (1997), with access to many more pigs and a larger experimental facility than was available to Taffs, interpreted their results as showing that migrating *A. suum* larvae are destroyed mainly from 14 to 21 days post-infection as they arrive back in the small intestine from the lungs. Somehow these findings explain why so many observers have recovered relatively few adult worms from pigs after giving large oral doses of infective eggs (Ogilvie and De Savigny, 1982; Stankiewicz *et al.*, 1992).

Ascaris larvae which survive the spell in the liver then move to the lungs for a few more days. In pigs' lungs, Taffs (1968) observed extensive oedema and emphysema which he attributed to hypersensitivity responses to *Ascaris* larvae. There exists a lively debate in the literature about the lung involvement of *A. lumbricoides* in humans (see below). An interesting consequence of the arrival of *A. suum* larvae in the lungs of pigs is the possibility that larval-induced lesions allow *E. coli* to invade, resulting in chronic pneumonia (Adedji *et al.*, 1989). In an experiment involving four pairs of piglets, embryonated eggs of *A. suum*, known to be infective to mice, were fed to the pairs of pigs either alone or together with an inoculum of a monoculture of *E. coli;* appropriate controls were used. Although very few pigs were available, Adedji *et al.* suggested that the pathogenic bacteria had been carried to the lungs by the migrating *A. suum*. The role of larval *Ascaris* as vectors for micro-organisms from the gut may be important. In a more convincing experiment with adequate numbers of mice, Tjornehoj *et al.* (1992) found that mice given aerosol

exposure to *Pasteurella multocida* while *A. suum* was in their lungs developed more severe pneumonia and septicaemia than *Ascaris*-free mice exposed in the same manner.

Other experimental work indicates that the dietary zinc intake of the host may influence events during the larval migratory phase. Laubach (1990) recovered larval A. *suum* from the livers and lungs of BALB/c mice given the same oral doses of infective eggs but fed on rations containing different amounts of zinc. More larvae were recovered from mice on low zinc rations during both primary and secondary infections. The liver eosinophil count was also reduced in the low zinc mice but not in the lungs.

2.1.2. *Larval* Ascaris lumbricoides

Until recently, very little was known about the pathology induced by larval *A. lumbricoides* in the human liver or whether this causes any recognizable disease. Presumably, similar observations would be made in human liver to those described for porcine liver when invaded by larval *A. suum*. However, an excellent and superbly illustrated account of the tissue phases of *A. lumbricoides* in humans has now been provided by Orihel and Ash (1995).

The early phase of ascariasis in humans is often reported to be associated with a distinctive type of pneumonitis (Little, 1985) which accompanies the migration of the larvae into and through the lungs. At an autopsy examination, larval *A. lumbricoides* were found in the bronchioles (Beaver and Danaraj, 1958) and they have also been retrieved from sputum (see Little, 1985). It is difficult to quantify the public health significance and extent of *Ascaris*-induced lung pathology. The abnormal single ingestion of massive numbers of *A. suum* eggs by four students clearly caused acute and life-threatening lung pathology (Phills *et al.*, 1972) and similar pathology would have been expected with a massive intake of *A. lumbricoides* eggs. In Saudi Arabia, where infection with *A. lumbricoides* occurs mainly from March to May, a pronounced pneumonitis and eosinophilia occur at the same time (Gelpi and Mustafa, 1967); these outbreaks of respiratory disease fit the description of Loeffler's syndrome.

Loeffler, during a study of tuberculosis, described a transient or seasonal syndrome of varying pulmonary infiltrates, mild to marked respiratory symptoms and peripheral eosinophilia (Spillman, 1975). The observations made later by Gelpi and Mustafa (1967) linked the seasonal aspect of Loeffler's syndrome to the seasonal aspect of ascariasis transmission and Loeffler (1956) had already concluded retrospectively that most of his cases of transient respiratory disorder might well have been due to larval *Ascaris* in the lungs. Strong support for the causal relationship between Loeffler's syndrome and the seasonal transmission of *A. lumbricoides* was obtained by Spillman (1975) who found only four cases of the syndrome after investigating 12 000 patients

in Colombia from communities where the prevalences of ascariasis were 25%, 36% and 82% and where transmission is not restricted to certain times of year.

An intriguing observation has been published by Doss and Tadros (1991) relating the presence of larval *A. lumbricoides* to changes in the activity of histidine decarboxylase [EC4.1.1.22] in the brain of infected guinea-pigs. Groups of three guinea-pigs each (control and infected) were sampled 10, 20, 30, 40, 50, 60, 70 and 80 days after the infected animals had each received a single oral dose of 3000 infective eggs of *A. lumbricoides*. Histidine decarboxylase activity was assayed by measuring the amount of substrate (histidine) remaining after a standard incubation involving homogenized brain tissue. The highest level of histidine decarboxylase in the tissue from infected animals was 1.888 ± 0.006 μmol/mg at 30 days post-infection as compared with 1.740 ± 0.012 from uninfected controls. Since samples of three animals were compared at each time point, statistical significance cannot be attached to this difference at 10 days post-infection and even then the biological significance is hard to understand. Doss and Tadros (1991) point out that histidine decarboxylase is induced by a variety of irritants and systemic stressors and speculate that it may play a role in the sensitization of humans to *A. lumbricoides* infection.

There is an interesting comment in their paper about the migratory behaviour of *A. lumbricoides*. In line with the current consensus they state that after about 10 days post-infection the larvae migrate through the respiratory passages to reach the oesophagus and then the small intestine. They also remark: 'We chose 80 days following infection as the suitable period of study, because during this time the larvae are migrating in the blood and lymphatic tissue and their effect will be maximal on the brain enzymes. In contrast, when the larvae were established in the intestine . . .'. Perhaps the time course of the *Ascaris*–guinea-pig association merits further study to clarify these points.

2.1.3. Ascaris *and Asthma*

Adult and larval *A. lumbricoides* and *A. suum* release extremely potent, volatile allergens (Coles, 1985; Kennedy, 1992) which may initiate the asthma-like symptoms sometimes associated with ascariasis. These symptoms may occur in both infected individuals and those handling the worms in the laboratory. The observation that elevated IgE levels accompany ascariasis gives credence to the connection between *Ascaris* infection and asthma. However, the relationship between *Ascaris*, allergy and asthma is still unclear and is complicated by the fact that the response of humans and various animals to *Ascaris* allergens has provided pharmaceutical companies with useful systems for developing and testing drugs designed to relieve asthma attacks.

Whether asthma attacks occur or not in *Ascaris*-infected people will depend on the variability of individual responses to worm allergens. In a long-term study of allergic responses of rhesus monkeys to antigen from *A. suum*, Patterson and Harris (1992) characterized three groups of monkeys: those with persistent and consistent IgE-mediated cutaneous and asthmatic responses to *Ascaris* antigen; those with cutaneous and airway reactivity to antigen in which the airway reactivity subsides in a manner analogous to spontaneous submission of human asthma; and those with cutaneous reactivity but no asthma. These types of variation in the form and severity of response have been described for humans. It is tempting to suggest that those humans with a tendency to develop asthma will also be susceptible to respiratory disease induced by exposure to *Ascaris* antigen especially if acquired by the aerosol route.

Perhaps continuous exposure to *Ascaris* infection in some way reduces the development of asthma as appears to be the case with Loeffler's syndrome (Spillman, 1975). Carswell *et al.* (1977), who investigated groups of asthmatic and healthy pre-school children in Tanzania where the overall prevalence of ascariasis is probably around 40% (see Crompton, 1989), did not find any evidence for *A. lumbricoides* as a cause of asthma. Aderele (1979) working with pre-school children in Nigeria found that, in a series of skin tests, sensitivity to *Ascaris* antigen was the commonest response observed although there was no consistent relationship between this sensitivity and the finding of eggs of *A. lumbricoides* in the children's stools.

2.2. Intestinal Stages

2.2.1. *Childhood Nutritional Status*

Seventeenth-century physicians were well aware of the deleterious health effects of *A. lumbricoides* on children. Tyson (1683) wrote '. . . that common roundworm which children usually are troubled with . . .' (Figure 2). Some 300 more years were needed before the World Health Organization endorsed the recommendation that in areas where the prevalence of mild–moderate underweight in children is greater than 25% and where parasites [intestinal helminths] are known to be widespread, high priority should be given to deworming programmes for treatment of parasites (ACC/SCN, 1989).

There is now agreement that under certain conditions infection with *A. lumbricoides* is associated with impaired growth and poor nutritional status in children. The topic has been extensively reviewed in recent years (see Crompton, 1985, 1992; Stephenson, 1987; Tomkins and Watson, 1989; Taren and Crompton, 1989; Thein Hlaing, 1993). Key papers in what has often proved to be a controversial debate are those by Gupta *et al.* (1977),

1. LUMBRICUS TERES, *or ſome* Anatomical *Obſervations on the* Round Worm *bred in human bodies. By* Edward Tyſon M. D. Col. Med. Lond. nec non. Reg. Societ. Soc.

HAving been ſo large in my *former* inſtance, in my *Diſcourſe* on the *Joynted-worm*, I intend to Contract my ſelf in *this*. Not that our preſent ſubject is ſcanty, or does not afford a ſufficient plenty of remarkable obſervations; But I choſe rather to ſelect what moſt ſuites our deſign. For to be exact and nice in all *particulars*, would require a juſt Treatiſe, and exceed the bounds I have at preſent ſet my ſelf.

I ſhall therefore here give the *Anatomy* of the *Lumbricus teres*, that common *Round Worm* which Children uſually are troubled with: and in this more particularly make my remarks upon the *Organs* of *generation* in both *Sexes*; and herein ſhew how vaſtly different they are from thoſe *parts* in the common *Earth Worms*, and it may be, moſt others. And withall I had deſigned, together with this, to have given the *Anatomy* of the *Earth Worm*, but ſince have altered my intentions: and at preſent ſhall refer to the account given of it by the famous Dr. *Willis*, reſerving my farther obſervations of it to another opportunity. This ſort of *Worm* by *Hippocrates* is named σειρὰς; by *Celſus*, *teres*; and is uſually about a foot long, or ſomething more, or leſs; but I have hitherto obſerved that the *Male* is generally leſſer than the *Female*: ſo that by their

Figure 2 Copy of the first page of Tyson's seminal paper on *Ascaris lumbricoides*. (Reproduced from Tyson, 1683.)

Willett *et al.* (1979) and Stephenson *et al.* (1980) each of which offered results indicating that successful anthelminthic chemotherapy to expel the intestinal stages of *A. lumbricoides* was accompanied by statistically significant gains in body weight and skinfold thickness. Later Thein Hlaing *et al.* (1991) demonstrated significant gains in both weight and height in children following treatment to remove *A. lumbricoides.* These studies involved communities in India, Tanzania, Kenya and Myanmar (formerly Burma). Provided that a suitable site is chosen, the study design is secure and adequate sample sizes are used, ascariasis can be shown to be a factor in the persistence of poor nutritional status in children of pre-school and primary school age.

Hadju *et al.* (1997) presented new findings and reviewed recent work carried out to investigate the relationship between infections with soil-transmitted helminths and the nutritional status of children. In their own study with urban slum dwellers in Indonesia, they demonstrated a significant increase in height gain in children who were regularly treated to remove *A. lumbricoides* as compared with children given a placebo. They pointed out the need to have an adequate sample size and to take account of confounding variables in the subsequent data analysis, including socio-economic status, geographical factors and concurrent infections.

Hadju *et al.* also drew attention to the results of studies by Stephenson *et al.* (1993a, b) which demonstrated significant weight gain in Kenyan schoolchildren and significant improvements in physical fitness (Harvard step test) and appetite following anthelminthic intervention. The studies involved *A. lumbricoides*, *T. trichiura* and hookworm and no attempt was made to separate their specific contributions to the overall improved performance. Appetite is not easily studied under field conditions so Stephenson *et al.* (1993b) devised a procedure involving the consumption of porridge made from maize meal (uji in Kenya) and known to have an energy content of 2709 kJ/l. Uji consumption was measured at certain times during the study and during the 4 month interval following anthelminthic treatment, the energy consumption of the treated group increased significantly ($P< 0.05$) over that of the placebo group when assessed by the objective test of uji consumption and by subjective tests based on interviews and questionnaires.

Hadju *et al.* fairly pointed out that amongst the more recent studies some do not observe any deleterious effect of ascariasis on the growth and well-being of children. For example, in a study involving 1402 children aged from 2 to 6 years old in Bangladesh, no significant growth improvements were observed in treated children although the prevalence of *A. lumbricoides* infection was shown to have been reduced from 71% to 6% as a result of the anthelminthic intervention (Rousham and Mascie-Taylor, 1994).

In practice, establishing the mechanisms whereby the intestinal stages of *A. lumbricoides* impair nutritional status is difficult due to the presence of

numerous types of other organism. It is not unlikely that one effect of *A. lumbricoides* is to facilitate the colonization or growth of micro-organisms. This fair point was forcibly made by Cooper *et al.* (1992) in a discussion of the apparent relationship between *A. lumbricoides* infection and temporary lactose intolerance described by Taren *et al.* (1987) in young children from Panama. Taren *et al.* compared amounts of exhaled breath hydrogen from *Ascaris*-infected and uninfected children given an oral lactose load after fasting. The results (Figure 3), which are consistent with the mucosal changes detected earlier by Tripathy *et al.* (1972) using biopsy techniques, are supported by results obtained from experiments involving *A. suum* in pigs (Forsum *et al.*, 1981) and were to be expected on the basis of a pilot study undertaken in Panama (Carrera *et al.*, 1984). The obvious involvement of *A. lumbricoides* in the lactose intolerance syndrome detected by Taren *et al.* (1987) was further confirmed when the production of breath hydrogen by dewormed children was found to have returned to the same level as that measured from uninfected children (Figures 3 and 4).

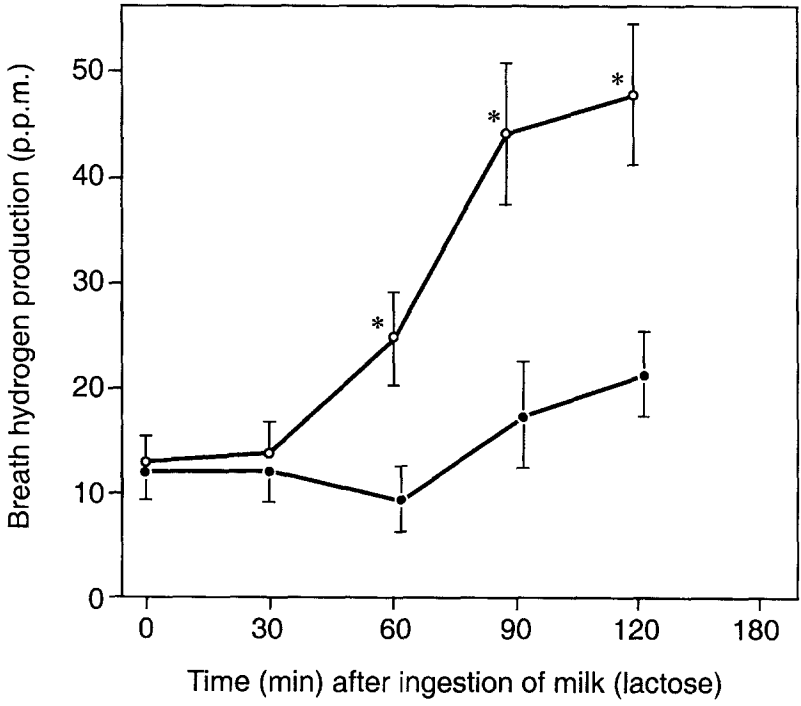

Figure 3 Breath hydrogen measurements from Panamanian children following the ingestion of lactose. (○ – ○)*Ascaris*-infected children [$n = 23$]. (● – ●) uninfected children [$n = 9$]. * = $P \leq 0.01$. (Reproduced from Taren *et al.*, 1987.)

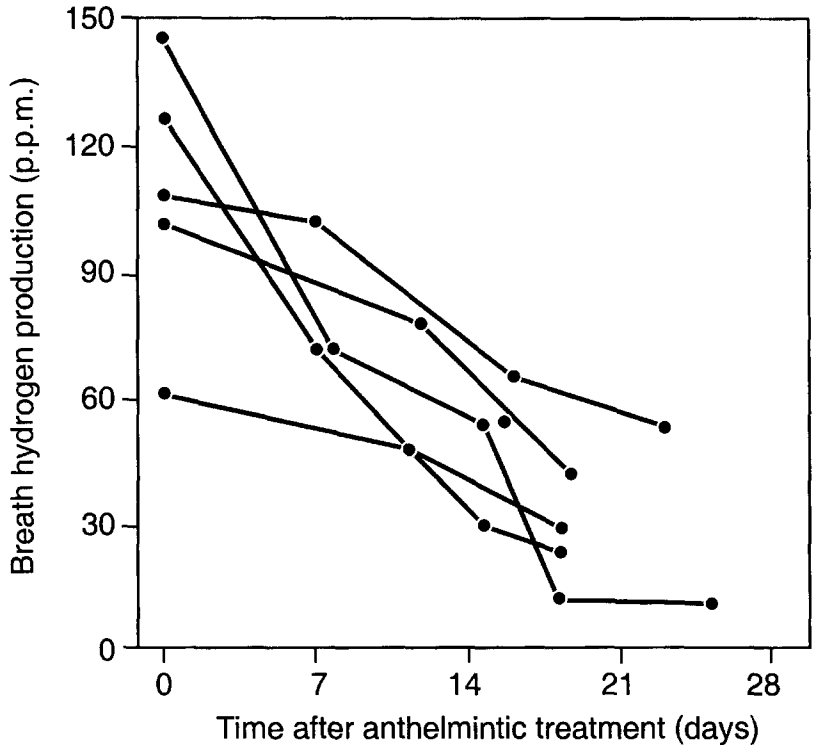

Figure 4 Breath hydrogen measurements from five Panamanian children following the ingestion of lactose after anthelminthic treatment to expel *Ascaris lumbricoides.* (Reproduced from Taren *et al.*, 1987.)

The role of *A. lumbricoides* in the vitamin A status of young children in West Sumatra, Indonesia, has recently been investigated by Jalal *et al.* (1998). The study design aimed at investigating whether intervention to expel the intestinal stages of *A. lumbricoides* with an anthelminthic drug (levamisole) would enable children to absorb more β-carotene from plant-based food sources as compared with similar children given a placebo. In some communities, food based β-carotene is a more readily accessible source of vitamin A than capsules and the costs of delivery can also be avoided or reduced. Jalal *et al.* found that the highest rises in serum retinol concentration (assumed to be evidence of increased dietary intake and absorption) occurred in children given meals containing additional β-carotene sources such as red sweet potato and extra fat and the anthelminthic treatment (Figure 5). An important consequence of these findings is the fact that in a public health programme intended

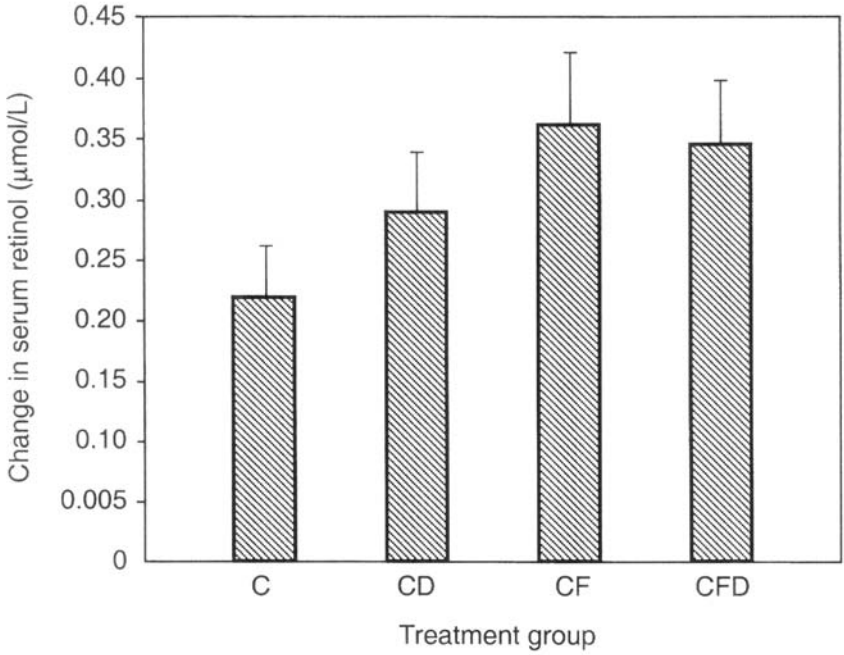

Figure 5 Comparison of changes in the serum retinol concentrations of Indonesian children following various interventions including anthelminthic treatment for ascariasis. C (n=39; basic meal + β carotene-rich food), CD (n=38; basic meal + β carotene-rich food + anthelminthic drug), CF (n=39, basic meal + β carotene-rich food + added dietary fat), and CFD (n=40, basic meal + β carotene-rich food + added dietary fat + anthelminthic drug.) Reproduced from Jalal *et al.*, 1998.)

to relieve vitamin A deficiency, there are likely to be extra benefits if steps are taken to offer regular anthelminthic treatment at the same time should endemic ascariasis be known to occur.

Ascaris lumbricoides clearly qualifies for the deworming recommendation (ACC/SCN, 1989) along with other species of soil-transmitted nematode. From the point of view of the health of children, it matters little to discover the exact role of *A. lumbricoides* in the generation of childhood malnutrition. Control programmes centred on the use of anthelminthic drugs are designed to deal simultaneously with the common species of common soil-transmitted nematode. A comprehensive bibliography covering recent studies of soil-transmitted nematode infections and their public health significance has been published by PAHO (1998).

2.2.2. *Cognitive Performance*

According to Warren *et al.* (1993) an important functional consequence of helminth infections in children is a reduced efficiency of education in imparting general intellectual skills. It is to be expected that children experiencing abdominal pain, nausea and digestive disturbance during an intestinal helminth infection will probably have a reduced attention span when present at school and may even miss a significant amount of schooling due to chronic illness. Demonstration of any such adverse effects of intestinal nematode infections on the intellectual development and cognitive performance of children is far from straightforward and is beset by numerous confounding variables (Connolly and Kvalsvig, 1993).

The most convincing early results in support of the proposition that intestinal nematode infections impair cognitive performance have been obtained from investigations focused on *Trichuris trichiura* in Jamaican schoolchildren (Nokes *et al.*, 1992a, b; Nokes and Bundy, 1993). This body of work, based on the well-tried experimental design of double-blind intervention with an anthelminthic drug controlled by means of a placebo, concluded that the expulsion of worms was associated with significant improvements in tests of auditory short-term memory and highly significant long-term memory. Furthermore, children with moderate to heavy infections of *T. trichiura* were observed to be less likely to comply with instructions or understand them adequately and to show higher rates of absenteeism. Simeon *et al.* (1994, 1995a, b) extended the study of *T. trichiura* in Jamaica and concluded that (1) moderate levels of infection with *T. trichiura* are associated with poor school achievement (Simeon *et al.*, 1994), (2) treatment for *T. trichiura* infection was more likely to benefit school performance in children of poor nutritional status (Simeon *et al.*, 1995a) and then (3) treatment of children with mild to moderate *T. trichiura* infections produces little benefit in cognition if they are adequately nourished (Simeon *et al.*, 1995b).

Raj *et al.* (1997) examined the effects of a single oral dose of albendazole on school attendance in a group of 249 early primary schoolchildren in Malaysia; *A. lumbricoides*, *T. trichiura* and hookworms were involved. Analysis of school attendance records was carried out for 60 days before and after anthelminthic treatment. There was no obvious improvement in the attendance rate following treatment of the children who had been infected when compared with those who had not. Also the correlation between intensity of infection and number of school days lost was weak. These results and those of Nokes *et al.* (1992a, b) again highlight the difficulty of making generalizations about the impact of infections based on a few studies in widely differing locations.

The same research effort as was made with *T. trichiura* has not yet been extended to investigating the effects or otherwise of *A. lumbricoides* alone on

the cognitive performance of primary schoolchildren. Boivin and Giordani (1993) have considered undernutrition, anaemia and intestinal helminthiases, including ascariasis, as a package of abuses to educational development and have concluded that efforts to increase school achievement levels require measures to improve the health and nutritional status of children. If further progress is to be made into the effect of *A. lumbricoides* and its relatives on cognitive performance perhaps young pre-schoolchildren as well as primary schoolchildren should be a group to be studied.

Two new studies to investigate the effect of soil-transmitted nematodes on cognitive function have been carried out with large numbers of children attending numerous primary schools in Indonesia (Hadidjaja *et al.*, 1998; Sakti *et al.*, 1999). Hadidjaja's team concentrated on the impact of *A. lumbricoides* and found that, following treatment with mebendazole, children showed significant improvements when tested for coding and in the use of coloured progressive matrices. The children also showed an improvement in learning ability, concentration and eye–hand movements 5 months after receiving mebendazole. Sakti's team were faced with children harbouring mixed infections. Their investigation involved pupils from 42 schools and, while recognizing that their results were associational in nature and that causation had not been demonstrated, they concluded that deworming is a beneficial intervention for helping education, particularly if hookworms are expelled.

2.2.3. *Complications*

Information about the variety and frequency of complications associated with the intestinal stages of *A. lumbricoides* is summarized in Table 3. These observations have been classified somewhat arbitrarily according to the location of the parasite in the patient when the observation was made. The references cited in Table 3 provide descriptions of typical signs, symptoms and case histories of the various lesions and complications. A simple analysis of the information given in Table 3 is set out in Table 4. The figures given by age and gender do not tally with the overall number of 4793 cases (Tables 3 and 4) because not all authors included the necessary details in their publications.

(a) *Complications of the gastro-intestinal tract.* Of the 4793 cases of complication under review, 3408 were associated with the gastro-intestinal tract, 2994 of which were cases of intestinal obstruction (Table 3). Intestinal obstruction due to a bolus of *A. lumbricoides* is regularly encountered in children from developing countries (Ukoli, 1984; Little, 1985). The observations in Table 3 suggest that *Ascaris*-induced intestinal obstruction occurs about 30 times more frequently in children than adults.

Table 3 The variety and frequency of complications attributed to the intestinal stages of *Ascaris lumbricoides*

Complication[a]	Cases	Literature example
Biliary system (1124)[b]		
Bile duct obstruction	1109	Khuroo *et al.* (1990)
Gall bladder	15	Dantas and Salles (1976)
Gastro-intestinal tract (3408)		
Appendicitis	100	Thein Hlaing *et al.* (1990)
Intestinal obstruction	2994	Ochoa (1991)
Intussusception	26	Kariholu *et al.* (1991)
Meckel's diverticulum	3	Pujari and Deodhare (1978)
Perforation (peritonitis)	151	D'Silva *et al.* (1980)
Stomach (haemorrhage)	3	Jacob *et al.* (1983)
Volvulus	131	Wiersma and Hadley (1988)
Hepatic abscess (100)	100	Bhave *et al.* (1978)
Pancreatitis (67)	67	Louw (1974)
Miscellaneous complications (94)[c]		
Brain (cerebral encephalitis)	50	Basu (1979)
Ear/eye/nose	15	Jain and Pahuja (1988), Vora *et al.* (1970), Maki (1972)
Heart	2	Da Costa Sobrinho *et al.* (1972)
Kidney	6	Bustamente-Sarabia *et al.* (1977)
Placenta	1	Chu *et al.* (1972)
Respiratory tract	9	Daya *et al.* (1982)
Spleen	1	
Thoracic cavity	1	Zamora Almeida (1976)
Tumoral form[d]	4	Bambirra *et al.* (1985)
Umbilicus	1	Ferrandez *et al.* (1972)
Urethra	2	Pamba and Musangi (1978)
Vagina[d]	3	Mali and Joshi (1987)
Total	4793	

[a] More detailed classifications of the diagnosis, pathology and treatment of the types of complication are given by Maki (1972), Khuroo *et al.* (1990).
[b] Chai *et al.* (1991) deal with an additional 1299 surgical cases of ascariasis (1198 biliary) treated in Korea (1955–1989); these results are not included in Tables 1 and 2.
[c] Maki (1972) provides an additional list of miscellaneous complications observed in Japan (1903–1958).
[d] May involve granulomas and 'tumours' formed around eggs of *A. lumbricoides*.

(b) *Bile duct obstruction.* Complications of the biliary system rank second to intestinal obstructions (Table 3) with a higher proportion apparently occurring in adults rather than children (Table 4). In a study of 500 patients experiencing hepatobiliary and pancreatic ascariasis in Kashmir, 133 were male while 367 were female (Khuroo *et al.*, 1990). The occurrences of hepatic abscess and pancreatitis are probably related to biliary complications induced

by the worm since the common bile duct provides a route from the small intestine to the biliary system, liver and pancreas. A recent analysis of cases of biliary-hepatopancreatic ascariasis in China indicated that migrations of *A. lumbricoides* from the gut into the bile duct account for about 12% of biliary disease in that country (see Zhou *et al.*, 1999).

A most comprehensive review of biliary ascariasis in Korea recorded that out of 1299 case histories of surgical ascariasis that occurred between 1955 and 1989, 1198 (92.2%) involved the biliary system (Chai *et al.*, 1991). Earlier, Maki (1972) pointed out that from 1903–1958 in Japan, 861 cases of biliary ascariasis (61.6%) were observed out of a total 1398 surgical cases. Chai *et al.* (1991) observed that in Korea, middle-aged men and women rather than children were more frequently affected. A similar conclusion about biliary ascariasis can be drawn from the summary of global data given in Table 4.

Table 4 Complications involving the intestinal stages of *Ascaris lumbricoides*

	Age		Gender		Deaths	
	Child	Adult	Male	Female	Child	Adult
Biliary system	1124	380	374	92	177	4
Gastro-intestinal tract	3408	2149	73	572	716	85
Hepatic abscess	100	52	6	17	15	13
Pancreatitis	67	28	26	11	15	1
Miscellaneous complications	94	77	15	50	41	9
Total	4793	2686	494	742	964	112

(c) *Development of complications.* Little information exists to explain how *A. lumbricoides* induces the variety of observed complications (Table 3). Both obstructions, particularly of the small intestine, and problems associated with the migrations of adult worms from the small intestine may be related to the intensity of the infection and should be investigated with this in mind. On average, children tend to harbour more worms than adults (Anderson, 1989). Factors that might cause large populations of worms to become tangled and form boluses will lead to obstructions. Factors that cause worms to migrate away from the intestinal population may lead to biliary and other complications. Ochoa (1991) suggested that fever, anaesthetic agents, drugs and vermifuges may prompt adult *A. lumbricoides* to migrate from the intestine while Pawlowski and Arfaa (1984) include peppery food in the list of migratory stimulants. High fever develops in patients with cerebral malaria and cases are known where adult *A. lumbricoides* have migrated from the small intestine to the pharynx so threatening to obstruct the airway (Wilairatana *et al.*, 1993).

Although 2994 cases of intestinal obstruction are listed in Table 3, reliable information about the worm burdens involved in the obstructions was found for only 32 cases (24 children and 8 adults). The values for the burdens from children range from 1 (Ghawss and Willan, 1990) to 796. In one case the intestinal obstruction in the intestine was caused by multiple adhesions around a single worm (Ghawss and Willan, 1990). A case of ileo-ileal intussusception due to a single worm was described by Yadava *et al.* (1974). Neither of these patients was reported to harbour any other adult *A. lumbricoides*. Of the 1109 reports of biliary complication (Table 3), 50 gave details of the numbers of worms involved. In 27 reports, the obstruction or lesion was attributed to one worm but each may have been a migrant from a larger population in the patient. One patient with an obstruction was found to harbour 92 worms and the bile duct of a patient treated by Pinus (1985) contained 69 *A. lumbricoides*.

It would have been useful if more observers had published the numbers of worms present in the patients that they treated. Extreme values, for example Pinus (1985) recovered more than 1000 worms from one patient, do not help with the development of guidelines for identifying the *Ascaris*-infected people who may be at risk from a life-threatening complication. A crude relationship exists between intestinal worm burden and faecal egg count. Given more detailed information about patient age, sex, complications and related worm burdens, simple risk indicators based on faecal egg counts might be made available for use in hospitals and clinics.

(d) *Anthelminthic treatment and complications.* Several authors have suggested that anthelminthic treatment may initiate some of the types of complication listed in Table 3. Bhagabati and Zaman (1972) recorded that intestinal obstruction in eight patients from Assam, India, developed after treatment with Santonin. Agugua (1983) implied that doses of vermifuge caused obstructing boluses of worms in 14 Nigerian patients. Carneiro (1987) attributed a case of intestinal obstruction in a Tanzanian child to the effect of piperizine citrate on *A. lumbricoides*. Aboh (1990) stated that ingestion of paralytic vermifuge contributed to a high incidence of intestinal obstruction and warned against purgation when dealing with the intestinal stages of *A. lumbricoides*. Brawley and Van Meter (1986) suggested that the migrations of *A. lumbricoides* might be drug induced. There is no doubt, however, that anthelminthic treatment serves to reduce the frequency of complications (Figure 6).

The possibility also exists that some individuals may be predisposed not only to certain levels of infection intensity with *A. lumbricoides* (Section 6.2) but also to induce the worms to migrate. Khuroo *et al.* (1990) investigated 500 patients with hepatobiliary and pancreatic complications associated with *A. lumbricoides*. After successful therapy with mebendazole, 76 patients (15%) were found to experience re-invasion of the biliary tree during a follow-up study lasting for 48 months.

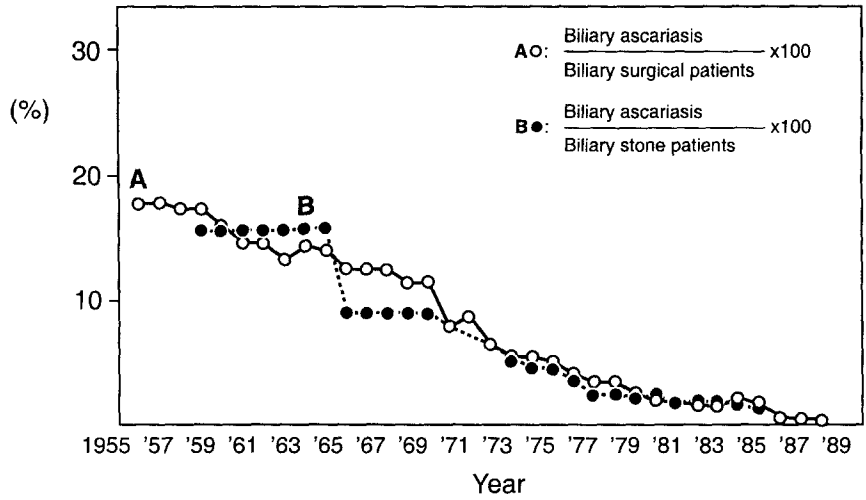

Figure 6 Decreasing pattern of the proportion of biliary ascariasis patients with regard to biliary surgical cases generally in South Korea. Control measures to reduce the impact of ascariasis were introduced nationally in the 1960s. (Reproduced from Chai *et al.*, 1991.)

(e) *Miscellaneous complications.* Fully developed *A. lumbricoides* have been retrieved by surgeons from a variety of ectopic sites (Table 3; Maki, 1972). It is usually assumed that adult worms migrated from the small intestine to the abnormal location but since larval *A. lumbricoides* undergo an obligatory but well defined tissue migration in human hosts, the possibility that some of the locations resulted from larval activity cannot be entirely ruled out. The granulomas or tumours formed around eggs of *Ascaris* spp. in visceral tissues (Bambirra *et al.*, 1985) and in the vagina (Mali and Joshi, 1987), indicate the presence of adult female worms in the patients at sometime. The encephalopathy described by Basu (1979) from 25 paediatric patients in Calcutta was based on meningism, convulsions, impairment of consciousness and abnormal EEG results. With the exception of a possible mild encephalitis from the presence of an arbovirus in one patient, intestinal infection with *A. lumbricoides* was identified as the incriminating factor in all cases.

(f) *Interference with surgical procedures.* Occasionally adult *A. lumbricoides* are found to have blocked tracheal tubes (Moyes and Rogers, 1971; Mimpress, 1972; Imbeloni, 1984), Ryle's tubes and naso-gastric tubes (Solanki *et al.*, 1975; Golz *et al.*, 1982) and gastrostomy tubes (Kabir and Afridi, 1972). The adult worms may also migrate from an intestinal site to an ectopic location following surgery. For example, Medeiros and Carvalho (1974) described a case of post-operative perforation of a neorectal bladder by *A. lumbricoides* in a patient following surgery to correct post-traumatic posterior urethral stricture.

Cases of this type are not included in Table 3 but their occurrence may remind surgeons working in areas where ascariasis is endemic to check for *A. lumbricoides* infection if procedures are likely to involve intubation.

Ascaris lumbricoides and *Ancylostoma duodenale* have also been implicated in the development of a lesion described by Trojan (1979) as *ulcus rodens* (rodent ulcer) in the eye. The ulcer is usually seen in elderly people and its aetiology is not well known (Dorland, 1985) but after studying 34 cases in Togo (Trojan, 1979) suggested that the lesion in question might arise as a result of an antibody reaction to toxins released by the worms.

2.3. Morbidity Rates

In making an assessment about the public health significance of ascariasis it is essential that decision makers have reliable evidence about morbidity rates. Competition for extremely scarce resources means that these should not be used unless there is a realistic chance of achieving sustainable benefits for the health of the community. Although there is abundant evidence to show that some individuals experience ill health associated with infection by *A. lumbricoides*

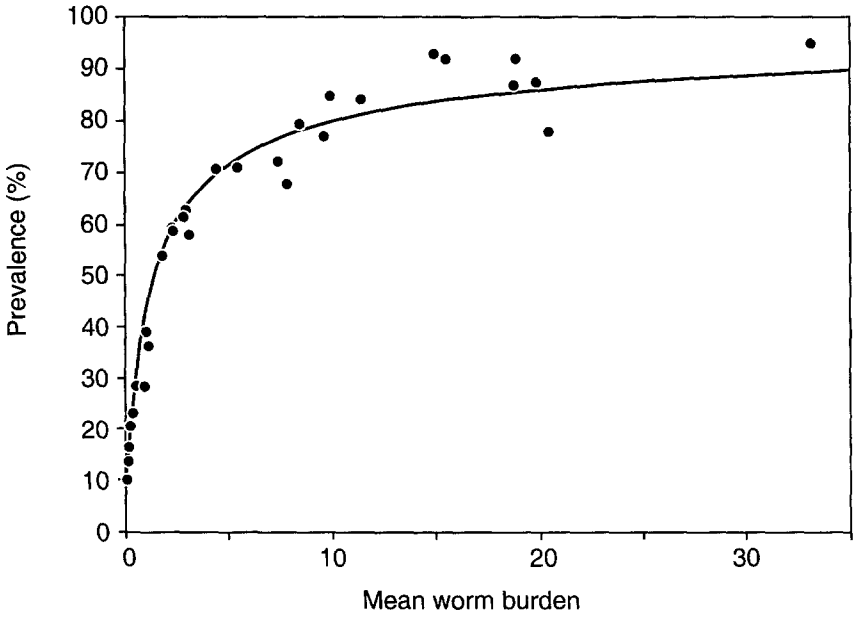

Figure 7 The relationship between the prevalence and mean intensity (worm burden obtained by expulsion chemotherapy) for *Ascaris lumbricoides*. (Reproduced from Guyatt and Bundy, 1991.)

(Section 2.2), this information does not constitute a quantifiable measure of the burden of disease.

Guyatt and Bundy (1991) have pioneered the development of a practical model for identifying communities at risk from disease due to intestinal helminth infections including *A. lumbricoides*. The model is based on the relationship between prevalence and mean worm burden (Figure 7). This relationship is non-linear and is determined by the frequency distribution of numbers of worms in the host population (Guyatt *et al.*, 1990). The relationship holds for a diverse number of infected communities across the world. Prevalence, based on detecting eggs of *A. lumbricoides* in stools, remains the measurement that can be made with reasonable accuracy given available resources in countries where ascarasis is endemic. The Guyatt–Bundy model can be applied to estimate the proportion of the community at risk of disease from ascariasis based only on measurements of prevalence. Guyatt and Bundy (1991) developed their model using published worm burdens based on counting worms passed in stools following anthelminthic treatment. Many studies have shown that egg counts, obtained by the Kato Katz technique (WHO, 1994) and expressed as eggs per gram of faeces (epg), provide a useful indirect measure of intensity of infection. In most cases, it can be assumed that the

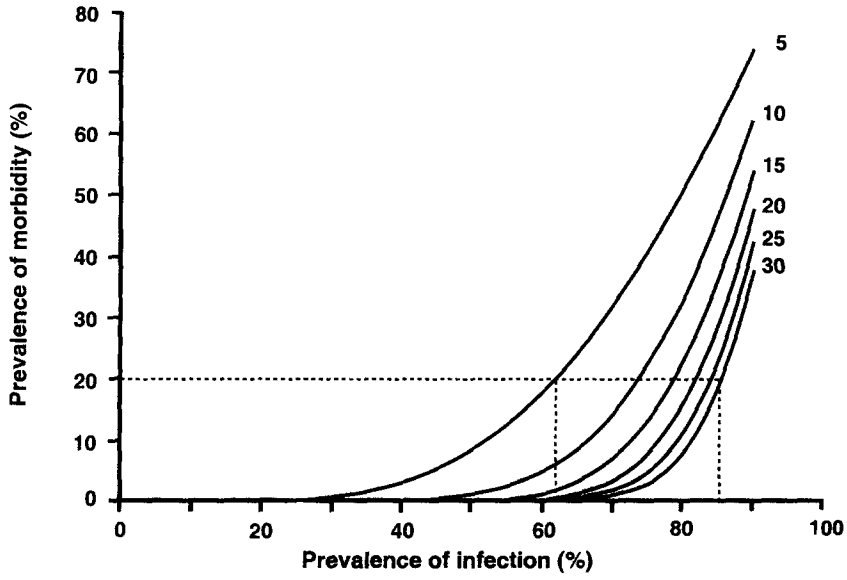

Figure 8 The relationship between predicted prevalence of morbidity and measured prevalence of *Ascaris lumbricoides* infection. The chart uses worm burdens of 5, 10, 15, 20, 25 and 30 worms as being responsible for initiating the morbidity. (Reproduced from Guyatt and Bundy, 1991.)

Table 5 Global estimates of potential morbidity due to infection with *Ascaris lumbricoides* set out by region (Estimates are given in millions)

Region	Population	Infections	Morbidity	Prevalence (%)	
				Infection	Morbidity
Sub-Saharan Africa	512	107	8[a]	21	1.61
			4.9[b]		
Latin America/Caribbean	441	182	26	41	5.99
			15.1		3.43
Middle Eastern Crescent	503	100	9	20	1.85
			5.5		1.1
India	850	198	22	23	2.63
			13.1		1.54
China	1160	568	91	49	7.84
		48.7			4.2
Other Asia and Islands	654	316	57	48	8.65
		34.7			5.31
Total	4120	1471	214	36	5.19
		122			2.96

[a] Upper number; estimated number of cases with developmental consequences e.g. impaired growth.
[b] Lower number; estimated number of cases with more serious consequences.

(Reproduced from Chan *et al.* [1994a] which should be consulted for technical details.)

Table 6 Estimates of potential morbidity due to infection with *Ascaris lumbricoides* set out by age class (Estimates are given in millions)

Age class (years)	Population	Infections	Morbidity	Prevalence (%)	
				Infection	Morbidity
0–5	553	139	5[a]	25	0.90
			0[b]		0.08
5–10	482	194	70	40	14.56
			55		11.4
10–15	437	180	60	41	13.79
			48		10.96
15+	2647	959	78	36	2.96
			19		0.71
Total	4120	1471	214	36	5.19
			122		2.96

[a] Upper number; estimated number of cases with developmental consequences e.g. impaired growth.
[b] Lower number; estimated number of cases with more serious consequences.

(Reproduced from Chan *et al.* [1994a] which should be consulted for technical details.)

higher the egg count the higher the number of mature female *A. lumbricoides* must be present in the small intestine. Guyatt and Bundy (1991) found that for *T. trichiura* and hookworms the relationship between prevalence and mean intensity based on egg counts followed the same pattern as that shown in Figure 7.

Morbidity during ascariasis generally depends on the number of worms present and a control programme should seek to reduce the worm burden and thereby reduce morbidity (Savioli *et al.*, 1992; WHO, 1996b); eradication is not the aim. It would be imprudent to propose a threshold worm burden that would be likely to initiate morbidity because the nutritional and immunological status of the host population will also be involved. The Guyatt–Bundy model incorporates a graphical chart (Figure 8) of the relationship between the predicted prevalence of morbidity and the measured prevalence of infection. For example, if health workers decide that 20% morbidity in a community is a high risk, then this state will prevail at a prevalence of 60% with a mean worm burden of five *A. lumbricoides* (Figure 8). There would appear to be a need to refine this model, strengthen the database, make adjustments using egg counts as measures of intensity and test its use under real conditions in areas of endemic ascariasis. Interestingly, Muller (1975) recorded that the fecundity of *A. lumbricoides* is equivalent to about 2000 epg per female worm per 24 hours.

The work of Guyatt *et al.* (1990) and Guyatt and Bundy (1991) was expanded by Chan *et al.* (1994a) who described a method for estimating the likely global morbidity due to *A. lumbricoides* and the other three common species of intestinal nematodes which infect humans. At the time of their study, Chan *et al.* assumed that the total number of *A. lumbricoides* infections was about 1472 million. Key statistics to emerge from their analysis were: (1) overall prevalence in the exposed population was 36%; (2) prevalence of morbidity in the exposed population was between 3 and 5.2%; (3) 122–214 million people were experiencing morbidity as a result of the infection. Epidemiological research (Anderson and May, 1991; Section 6) has found that on average children harbour higher worm burdens than adults and so are likely to suffer more morbidity. Chan *et al.* applied this finding in their analysis and showed children (aged 5–15 years) were at greater risk of morbidity during ascariasis than other groups in the community. Summaries of the potential morbidity due to ascariasis in regions of the world and by age class are set out in Tables 5 and 6 respectively.

2.4. Mortality Rates

Mortality rates associated with a disease such as ascariasis are extremely difficult to estimate with any degree of confidence. The disease is endemic in regions where many infected people will rarely have access to health care and

where statistical information is not always recorded on a regular basis. The problem of estimating ascariasis-related mortality rates has been reviewed by Pawlowski and Davis (1989) who designed a questionnaire which was distributed to selected hospitals in Brazil, China, Egypt, Myanmar (formerly Burma), Nigeria, South Africa and South Korea. None of these countries falls into the class of 'least developed' (UNICEF, 1998) where record keeping might be expected to be least efficient.

During the past 20 years a miscellany of observations has been published to draw attention to the mortality rate associated with ascariasis. Walsh and Warren (1979) produced a global figure of 20 000 deaths per year. Pinus (1985) recorded a fatality rate of 9.3% among 454 cases admitted with surgical complications to a hospital in Sao Paulo, Brazil. Thein Hlaing *et al.* (1987) reported that six children died annually from ascariasis in Rangoon (now Yangon) Children's Hospital. Of the 4793 cases referred to in Table 3, 210 died, 112 being children, 38 adults and 60 being of unknown status (Table 4). These figures suggest that about three times as many children die from acute ascariasis as adults, presumably from gastro-intestinal complications. The death rate for adults suffering from biliary complications (Table 4) would appear to be higher than that observed for children. This overall death rate (210/4793) seems to be extremely high and should not be applied generally, particularly since the data extend over a long time span. From the results of their survey, Pawlowski and Davis (1989) concluded that as many as nine children per 1000 admitted to hospital with acute ascariasis might die and that perhaps 100 000 people might die annually.

All the cases listed in Table 3 had been admitted to hospital and of these the form of treatment of 1886 is described with 1138 requiring surgical intervention (laparotomy, enterotomy, resection, manual advancement, endoscopy) and 748 being relieved by non-operative, conservative management (electrolyte replacement, nasogastric suction, anthelminthic drugs and antibiotics). The cost of this health care would be quite considerable for a developing country. Would its expenditure on community control programmes have been more effective and would more lives have been saved? Evidence from South Korea has shown a marked decline in the number of cases of biliary ascariasis as a proportion of all biliary surgical cases since the introduction of control measures in the community (Chai *et al.*, 1991; Figure 6).

3. IMMUNOLOGY AND ALLERGIC RESPONSES

There exists little direct evidence to show that humans mount a strong protective immune response against *A. lumbricoides* but a considerable body of circumstantial evidence indicates that some degree of protection occurs, the

most convincing being the fact that on average the intensity of infection is lower in adults than in children living in the same environment (Section 6.1) and that individual predisposition to the degree of intensity of infection is also well documented (Section 6.2). It is difficult to account for these features of the human–*Ascaris* association without recourse to partial protective immunity influenced by individual variation in the extent of the protection achieved. Understandably, experiments with human subjects involving controlled infections cannot be undertaken. Experiments with pigs and *A. suum* (or *A. lumbricoides*) are expensive and even with what seems to be a closely related host–parasite system some caution must be exercised before extrapolation to the human situation is made. Many experiments have been carried out using laboratory mice as hosts to study the tissue phase of an *Ascaris* infection; even greater caution is needed when extending the significance of these results to human hosts.

3.1. Animal Hosts

3.1.1. *Laboratory Mice*

Infective eggs of *A. suum* (and *A. lumbricoides*) readily hatch in mice of various strains releasing larvae which migrate to the liver and lungs (Eriksen, 1981). As an example of an informative experiment Eriksen gave each of 790 mice (some were congenitally athymic and some had normal thymic function) an oral dose of 1000 infective eggs of *A. suum*. Groups of mice were sampled at regular intervals post-infection to follow the course of the infection and to measure indicators of immune activity. Many of the mice also received a challenge dose of 1000 infective eggs 4 weeks after the first dose. The results showed that the elimination of larval *A. suum* during primary and secondary infections is influenced by thymus-dependent immune responses. For example in athymic mice more larvae reach the abdominal cavity, the lungs and gut than in thymus-bearing mice. Also in the athymic mice, the eosinophilia characteristic of ascariasis was not pronounced, relatively few white spots appeared on the liver and serum antibody was not detected until 15 days post-infection in the primary infection compared with 11 days post-infection for mice with a functional thymus.

The immune effector mechanisms responsible for the results obtained by Eriksen (1981) and other workers who have studied the mouse–*Ascaris* system are not well known. Grencis (1997) reviewed the results of studies involving long established strains of laboratory rodents and long established laboratory isolates of their habitual intestinal helminth infections, including as an example rats and *Nippostrongylus brasiliensis*. This body of work shows that in immunocompetent animals worm expulsion from the gut requires the

generation of a Th2-mediated response. According to Grencis the cytokines secreted by these cells would control intestinal mastocytosis, eosinophilia and IgE production. Although *A. suum* may not be expelled from the intestine of mice in the manner of the infections discussed by Grencis, it seems reasonable to predict the involvement of Th2-cell mediated responses. However, the effector mechanisms differ between host–helminth relationships, between stages in the life history of a specific helminth and between primary and secondary infections with the same species of helminth (Grencis, 1997).

The marked production of IgE during an *Ascaris* infection has proved difficult to explain (Pritchard, 1993). Lee and Chang (1995) found that the body fluid of *A. suum* contains a B-cell mitogen which stimulates spleen cells, isolated from BALB/C mice and maintained *in vitro*, to proliferate as measured by the uptake of tritiated thymidine. The B-cell proliferation also required the presence of murine accessory cells; these were shown to be macrophages obtained from peritoneal washings. Lee and Chang proposed that B-cells in the mouse when influenced by IL4, a product of Th-2 cells, would switch to produce IgE. There is much evidence to show that IL4 is involved in IgE production *in vivo* (Finkelman *et al.*, 1988) but why should so much IgE be secreted by hosts harbouring *Ascaris*? Perhaps the parasite releases an IL4-like molecule which diverts the host to release much irrelevant antibody. With that idea Lee and Chang (1995) speculated that IgE production during ascariasis is a manifestation of how *Ascaris* survives in immunocompetent hosts. Earlier, Pritchard (1993) had examined the consequences of the production of the apparently superfluous IgE production by the host during a helminth infection and suggested that disproportionately high levels of non-parasite specific IgE could be beneficial to a parasite if such molecules were to saturate receptors at the surface of mast cells. Similarly, Pritchard argued that ADCC might be impaired if non-specific IgE were to saturate receptors at the surface of eosinophils and macrophages.

3.1.2. *Domesticated Pigs*

If the excessive IgE production is somehow related to the survival of *Ascaris* in immunocompetent hosts it must be reconciled with the fact that under laboratory conditions patent infections can be difficult to establish in pigs, especially when exposed to single oral doses of large numbers of infective eggs. For example, Stankiewicz *et al.* (1992) gave each of 10, 4-week-old pigs a single oral dose of 10 000 *A. suum* eggs. Seven days later, numerous milk spots were present in the livers and many larvae were present in the lungs in four of the pigs. Post-mortem examinations of the remaining six pigs, 60 days post-infection, produced two adult female *A. suum* from one pig only. Forsum *et al.* (1981) recovered an average of 74 adult *A. suum* from pigs

given three doses, each of 200 eggs, on alternative days. Under natural conditions infections of *Ascaris* in both pigs and people will be acquired by a gradual and erratic fashion, a view confirmed by finding *Ascaris* intestinal stages of both sexes at different stages of development and size (Seo and Chai, 1980; Petkevicius *et al.*, 1996).

Under experimental circumstances, domesticated pigs are well known to acquire some degree of protective immunity to *A. suum* through experiencing repeated infections (Kelley and Nayak, 1964; Taffs, 1968; Eriksen, 1981). Stronger immunity is developed by older than younger pigs. Pigs that were 2 weeks old when first exposed to *A. suum* eggs contained about 26 000 larvae in their lungs when challenged as compared with pigs which were 6 weeks old on first exposure and contained about 3500 larvae on challenge (Kelley and Nayak, 1964). Also under experimental conditions, suckling piglets have been passively protected against *A. suum* by giving them either immune serum or colostrum (Kelley and Nayak, 1965a, b). The passive immunity observed by Kelley and Nayak points to the importance of a humoral response under the conditions they used.

Urban and Tromba (1982, 1984) demonstrated that pigs exposed to UV-irradiated 'infective' eggs of *A. suum* developed protective immunity when challenged with subsequent doses of non-irradiated infective eggs ($P<0.05$; experimental vs. control pigs). The conclusion was that larval *A. suum* must be sources of protective antigens and so Urban and Romanowski (1985) carried out a series of experiments to see if products derived from egg hatching fluid (EP; egg products) or larvae maintained *in vitro* (ESP; excretory – secretory products) could be used to immunize pigs. Their experimental designs and results are summarized in Table 7. These results demonstrate that pigs can develop some degree of protective immunity against *A. suum*. Urban and Romanowski (1985) also observed that relatively mild milk spot reactions were present in challenged pigs initially immunized with non-parasite material (Experiment 1, Table 7) as compared with prominent reactions in challenged pigs initially given parasite products. This result also supports the view that many migrating larval *Ascaris* are likely to perish in the tissues if the host is regularly ingesting infective eggs.

Since these studies, research into the pig–*Ascaris* association has focused mainly on nutrition, husbandry and population biology (Nesheim, 1985; Roepstorff and Nansen, 1994; Roepstorff and Murrell, 1997; Boes, 1999). Some of the recent results, however, cannot be explained unless an immunological dimension is included. For example, Eriksen *et al.* (1992) arranged 84 pigs into three groups to explore the results of three infection procedures. Pigs of group 1 (high trickle) were each offered 500 infective eggs of *A. suum* twice weekly, those of group 2 (low trickle) 25 infective eggs twice weekly and those of group 3 (control) were not offered eggs. This system began when the pigs weighed 25 kg and stopped when they reached 90 kg (10–16 weeks); pigs

Table 7 Protective immunity against *Ascaris suum* in pigs (after Urban and Romanowski, 1985)

Experiment 1: Results following immunization with soluble egg and larval products

Group (no. of pigs)	Immunization[a] Day 1	Day 7	No. of larvae recovered[b]	Percentage protection
I (5)	OA (po)	OA (po) KLH (ip)	2358 ± 340*,[c]	–
II (4)	EP (po)	EP (po) L2,3 + L3,4 ESP (ip)	897 ± 343**	62

Experiment 2: Results following immunization with excretory–secretory products and UV-irradiated eggs

Group (no. of pigs)	Immunization[a] Day 1	Day 7	No. of larvae recovered[b]	Percentage protection
I (7)	OA (ip)	–	2439 ± 193*,[c]	–
II (7)	L3,4ESP (ip)	–	1771 ± 275**	27
III (7)	L3,4ESP (ip) + UV-eggs (po)	–	1611 ± 162**	34
IV (7)	L3,4ESP (ip)	UV-eggs (po)	484 ± 76***	80

Experiment 3: Results following immunization with increasing doses of excretory–secretory products and UV-irradiated eggs

Group (no. of pigs)	Immunization[a] (ESP dosage in μg/kg body wt) Day 1	Day 7	No. of larvae recovered[b]	Percentage protection
I (6)	OA (90)	UV-eggs	3317 ± 244*,[c]	–
II (6)	L3,4ESP (30)	UV-eggs	2253 ± 349**	32
III (6)	L3,4ESP (90)	UV-eggs	2312 ± 392**	30
IV (6)	L3,4ESP (270)	UV-eggs	2130 ± 202***	36
V (6)	L3,4ESP (<10,000M_r)	UV-eggs (po)	2732 ± 300**	18

Explanatory notes

[a] Immunization schedule: ip, intraperitoneal; po, *per os*; OA, ovalbumin; KLH, keyhole limpet haemocyanin; EP, egg-derived products; ESP, excretory–secretory products (larval stages indicated); UV-eggs, 30 000 eggs of *A. suum* exposed to 400 μW-min/cm^2 UV light. Refer to original paper for details of the preparation and characteristics of ESP doses.

[b] Pigs received a challenge dose of 10 000 infective eggs of *A. suum* 21 days after immunization and larvae were retrieved from the lungs 7 days later.

[c] Mean ± SE; means with same number of asterisks are not significantly different. $P<0.05$ taken to be significantly different.

from each group were examined at regular intervals during the course of the infection. Eriksen *et al.* confirmed the common experience that, regardless of the dosing regimen, the numbers of *A. suum* that arrive in the small intestine is generally erratic and independent of the intake of infective stages. Some 3 weeks after the start of the experiment, the numbers of *Ascaris* in the small intestine of pigs from the high- and low-trickle groups reflected the numbers of eggs to which the pigs had been exposed. Thereafter, the numbers of *Ascaris* in the intestine were observed to be unrelated to the dose and to be essentially all adults after 6 weeks even though there was adequate time for more infective eggs to have been swallowed, for larvae to have migrated through the liver and lungs and for immature worms to be developing in the gut. It is difficult to explain these important results without concluding that some time after initial exposure of the host to *Ascaris* antigen(s), larval stages are killed in the tissues and immature worms are killed in the gut. Additional variability, including the regularly observed overdispersed frequency distribution of numbers of worms per pig (Roepstorff *et al.*, 1997), will be expected to stem from individual host genetic variability.

3.2. Human Hosts

Immunological aspects of the human–*Ascaris* association are generally commensurate with those of the pig–*Ascaris* association; humans also appear to develop some degree of protective immunity. This can be inferred from the analysis of the results of epidemiological surveys (see Anderson and May, 1991) and is supported by clinical investigations. Jones (1977) studied communities in Papua New Guinea that were exposed to a range of infective stages and showed varying intensities of infection with *A. lumbricoides*. By means of an indirect haemagglutination test, Jones found higher antibody titres in people enduring more frequent and intense contact with the parasite. Jones suggested that in an area where continuous reinfection must occur, individuals with low egg counts and high antibody titres had developed immunity; relatively few sexually mature adult worms were able to develop in such hosts. Another line of evidence in support of some level of protective immunity was obtained by Thein Hlaing *et al.* (1987) who found that children kept worm-free by means of anthelminthic treatment gained higher worm burdens than children who had had some treatment but not enough to prevent the establishment of sexually mature adult worms. These studies indicate that steady exposure to larval and adult stages of *A. lumbricoides* elicits some immunity.

A marked heterogeneity has been observed in the antibody responses of people to *Ascaris* antigens. Haswell-Elkins *et al.* (1989) studied the antigen recognition profiles of people from whom serum had been prepared after

worm burdens had been determined by expulsion chemotherapy using pyrantel pamoate (Elkins *et al.*, 1986). Larval excretory–secretory antigens were prepared from *A. lumbricoides* collected from people living in the same community. Results obtained from 60 participants showed that the antibody response profile followed the age-related intensity pattern. Individuals with high worm burdens, who tended to be children, showed high antibody levels. Haswell-Elkins *et al.* conclude that the higher antibody levels in children probably result from greater exposure to *A. lumbricoides* because their standards of personal hygiene are lower than those of adults. This view is in effect supporting a behavioural rather than an immunological explanation for the universally recorded decline in intensity with host age (Section 6.1).

Characteristically high concentrations of IgE are also detected together with eosinophilia during ascariasis in humans. These events are typical of allergic responses and Kennedy (1992) has discussed their significance and sought to explain the nature of allergens. Some molecules, released either by *A. lumbricoides* or by the host or by both partners, during the course of the infection stimulate the massive production of IgE. Hagel *et al.* (1993) investigated total IgE and *Ascaris*-specific IgE levels in 98 Venezuelan children known to be infected with *A. lumbricoides* at the start of the study. Children were given anthelminthic treatment; total IgE levels then fell in all the children and continued to fall in those who did not become reinfected but rose in those who became reinfected. The reverse pattern was detected for *Ascaris*-specific IgE levels with reinfected children appearing unable to maintain high levels of *Ascaris*-specific IgE. This work argues in favour of IgE functioning to protect both host and parasite. Although schistosomes represent an entirely different group of helminths from ascarids and will present the human host with an entirely different set of antigens, it is now accepted that IgE has a protective role against infection with *Schistosoma haematobium* (Hagan *et al.*, 1991).

A wide variety of antigens has been characterized from both larval and adult *A. lumbricoides* and *A. suum* (Kennedy and Qureshi, 1986; Knox and Kennedy, 1988; Kennedy *et al.*, 1987). Perhaps the most interesting of these is a 25 kDa protein molecule isolated from the body fluid of larvae and adults of both *A. lumbricoides* and *A. suum* and known as ABA-1 (McGibbon *et al.*, 1990). This molecule is the major protein in the body fluid and its allergenic activity is shown by its ability to cause the release of histamine from sensitized mast cells *in vitro*. The same allergenic molecule has been isolated from the body fluid of adult *A. lumbricoides*.

Current knowledge of the immune response to *A. lumbricoides* offers little prospect at present for the development of a vaccine. However, isolation of a purified allergenic molecule by McGibbon *et al.* (1990) is a useful step in the procedure for vaccine development. Commercial opportunities for a vaccine

may emerge with pig husbandry in mind if consumers strengthen demand for organically-produced pork devoid of exposure to anthelminthic drugs and chemical additives to feedstuffs. Understanding immunity during ascariasis remains important if only to ensure that it is not undermined by the use of anthelminthic drugs during control programmes.

4. BIOLOGY OF *ASCARIS LUMBRICOIDES*

4.1. Systematics and Host Specificity

The widely accepted systematic schemes for the classification of *A. lumbricoides* are set out in Table 8. There is still no overriding consensus about whether the nematode body plan is distinct enough to merit phylum status and there remains enormous scope for the phylogenetic speculation offered by the assemblage of animals which are recognized as pseudocoelomates (Willmer, 1990). Russian authorities have tended to place the Nematoda as a class in the phylum Nemathelminthes and to divide the order Ascaridida into more than one suborder. This arrangement can be compared with those in Table 8 by consulting Mozgovoi (1953), who also provides a comprehensive description of the anatomy of *Ascaris* spp. and their relatives.

Table 8 Common schemes for the classification of *Ascaris lumbricoides*

Phylum	Nematoda	Aschelminthes
Class	Secernentea	Nematoda
Subclass		Secernentea
Order	Ascaridida	Ascaridida
Superfamily	Ascaridoidea	Ascaridoidea
Family	Ascarididae	Ascarididae
Subfamily		Ascaridinae
Genus	*Ascaris*	*Ascaris*
Species	*lumbricoides*	*lumbricoides*

The genus *Ascaris* comprises 16 species according to Yamaguti (1961) and 13 species according to Skryabin *et al.* (1991) (Table 9). Details of naturally and experimentally infected host species reported to harbour *A. lumbricoides* can be traced through references included in Table 9. Knowledge of the host specificity of *A. lumbricoides* is important in case some species may serve as reservoir hosts in some locations and so impede the success of control measures. At present, *A. lumbricoides* appear to show remarkably high specificity for humans and it remains to be seen whether any other species of host is able

Table 9 Host specificity of species of *Ascaris*

Species
Ascaris lumbricoides (type species) Linnaeus (1758) from humans worldwide; also in *Alouatta palliata* (mantled howling monkey), Costa Rica (Stuart *et al.*, 1990); *Canis familiaris* (domestic dog), Japan• (Leiper, 1915); *Callosciurus pygerythrus* (Irrawaddy squirrel), Asia (Baylis and Daubney, 1922); *Gorilla gorilla* (gorilla), laboratory (Orihel, 1970); *Hylobates agilis* (agile gibbon), Singapore (Dunn and Greer, 1962); *Hylobates lar* (gibbon), laboratory (Orihel, 1970); *Macaca arctoides* [= *M. speciosa*] (bear macaque), Asia (Kobayashi, 1925); *Macaca mulatta* (rhesus monkey), India • (Remfry, 1982); *Pan paniscus* (pygmy chimanzee or bonobo), Congo• (Stam, 1960); *Pan troglodytes* (Chimpanzee), laboratory (Orihel, 1970); *Ratufa indica* (Indian giant squirrel), Asia (Baylis and Daubney, 1922); *Sus domesticus* (domestic pig), see Galvin (1968)
A. brevispiculum in *Apodemus chevrieri* [= *A. agragrius*] (species of wood mouse), former USSR•
A. castoris in *Castor fiber* (Eurasian beaver), Europe
A. cebi in *Cebus capucinus* (white-throated capuchin monkey), Honduras to Colombia
A. columnaris in *Mephitis mephitis* (striped skunk), *Mustela erminea* (stoat), *Procyon lotor* (raccoon) and related mammals from N. America
A. dasypodina in *Dasypus gymnurus* (armadillo), Paraguay•
A. devosi in *Martes americana* (American marten), N. America
A. hippopotami in *Hippopotamus amphibius* (hippopotamus), Sub-Saharan Africa
A. joffi in *Citellus pygmaeus* (little souslik), Kazakhstan•
A. laevis in *Marmota monax* (woodchuck) and *Citellus* [= *Spermophilus*] parryii (Arctic ground squirrel), N. America
A. ovis in *Ovis aries* (domestic sheep) and *Saiga tatarica* (Saiga), Australia, N. America, UK and former USSR
A. phacocheri in *Phacochoerus africanus* (wart hog), Democratic Republic of the Congo•
A. schroederi in *Ailuropoda melanoleuca* (giant panda), New York Zoo via Sichuan, P.R. China•
A. spalacis in *Spalax* [=*Nannospalax*] *leucodon* (species of blind mole-rat), Balkans
A. suricattae in *Suricata suricatta* (Meerkat), Transvaal, Republic of S. Africa•
A. suum in *Sus domesticus* (domestic pig), worldwide; *Sus scrofa* (wild boar), worldwide (Eslami and Farsad – Hamdi, 1992); *Bos taurus* (domestic cattle), Sweden (Roneus and Christensson, 1977); humans, see Lysek (1961); *Erythrocebus patas* (red monkey) laboratory (Urban *et al.*, 1998)
A. tarbagan in *Marmota sibirica* (Siberian marmot), Siberia•

1. Scientific and common names of wild hosts taken from Corbet and Hill (1991).
2. Scientific and common names of domestic hosts taken from Clutton-Brock (1987).
3. • Place of detection; wider distribution not given.
4. For *A. lumbricoides* and *A. suum* there is evidence that the host species harboured mature worms. References are cited in the entries. In many reports authors express their concerns about whether they were dealing with *A. lumbricoides* or *A. suum*.
5. Detailed chronological record of the experimental demonstration that *A. lumbricoides* is a habitual endoparasite of humans is given by Grove (1990).
6. Yamaguti (1961) considers *A. ovis* to be a synonym of *A. lumbricoides* and does not list *A. cebi* which was described in 1921.
7. Skryabin *et al.* (1991) do not list *A. brevispiculum*, *A. devosi*, *A. laevis* and *A. spalacis*.
8. References to the species of *Ascaris* mentioned above other than *A. lumbriocoides* and *A. suum* may be traced through Skryabin *et al.* (1991) and Yamaguti (1961).

to support the parasite while it attains sexual maturity and produces fertile eggs at a level needed to maintain its survival. For example, Rausch and Tiner (1948) found specimens of immature *Ascaris* sp. in various squirrels from locations in the USA and, despite identifications by earlier workers suggesting that such ascarids were likely to be *A. lumbricoides*, Rausch and Tiner doubted whether that ascarid could mature in squirrels. Mature female *A. lumbricoides* or *A. suum*, sometimes measuring over 450 mm in length (DWTC, personal observations), would present a serious obstruction in the small intestine of a squirrel. In contrast, Juniper (1978) found a species of *Ascaris* that had attained maturity in Canadian Black Bears (*Ursus americanus*). This is much more likely to have been either *A. lumbricoides* or *A. suum*.

The information set out in Table 9 is not in any way quantitative regarding the significance of the non-human hosts for *A. lumbricoides*. Some authors indicate that the finding of *A. lumbricoides* in non-human primates results from contact between them and human settlements or zoos (Orihel, 1970; Nesic *et al.*, 1991; Stuart *et al.*, 1990).

The larvae of *A. lumbricoides* and *A. suum* show a remarkable propensity to hatch in the gut and migrate through the tissues to at least as far as the lungs in a variety of non-ruminant and ruminant mammals. Evidence for this generalization is available from Table 9 and from table 3 in Crompton (1989) which cites references to the experimental exposure of cattle, dogs, goats, guinea-pigs, mice, monkeys, rabbits and rats to infective eggs of *Ascaris* obtained from either humans or pigs. Gaur and Deo (1972) successfully infected lambs with *Ascaris* under experimental conditions and respiratory complications were observed. Examples of humans being exposed to *Ascaris* of pig origin and *vice versa* are also well known (Crompton, 1989). The determinants of host specificity, therefore, would appear to involve the parasite's response to the conditions prevailing in the host's gut, unless the tissue migration in a refractory host irreparably damages the parasite.

4.2. Life History

There might appear to be little new to be learned about general aspects of the life history of *A. lumbricoides*, knowledge of which is regularly reviewed (see Crompton, 1989, 1997). However the significance of certain aspects of the behaviour and development of the parasite remain poorly understood particularly regarding its tissue migration on entering its natural host. The larvae of several species of animal parasitic nematode seem to migrate through the tissues for no obvious purpose. In the case of *A. lumbricoides*, why should this parasite, which enters its host by the alimentary tract, immediately leave the site to which it must return to reproduce? Why should *A. lumbricoides* expose

its larval stages to the immune surveillance and effector mechanisms of its host only to return to the more congenial immunologically secluded habitat in the gut from which its transmission stage is adapted to escape?

Smyth (1994) summarized the view that this tissue migration is essentially a phylogenetic throwback and that such nematodes as undergo it are reliving part of a life cycle that once occurred in another host. Read and Skorping (1995) have argued that such migrations may not in fact be evolutionary baggage and, despite the expected loss of larvae during the migration, a trade off should ensue, such as increased reproductive fitness for those who return to and mature in the gut. Read and Skorping tested this notion by assembling development data for 700 species of nematode which live as adults and juveniles in mammalian hosts. Attention was paid to the growth (length, width, volume), longevity and fecundity of female nematodes and the dimensions of their eggs. With few exceptions, taxa developing in the tissues were found to grow faster and have larger bodies as adults than their closest relatives living only in the alimentary tract. Female body size is strongly linked to fecundity in nematodes (Skorping *et al.*, 1991). This evolutionary trade off can be applied to *A. lumbricoides;* losses during tissue migration are compensated for by the presence of larger females producing more eggs in a site of easy egg release from the host.

Much information has recently been obtained by Murrell *et al.* (1997) about the tissue migration of *A. suum* in pigs. These findings are likely to be relevant to the human *Ascaris* relationship because they provide insight into the nature of the immune response (Section 3.1.1). Fourteen female pigs, bred specially for the purpose and known to be free from infection with *A. suum,* were each given an oral dose of 500 000 eggs and were then killed in pairs and examined for larvae at 3, 6, 9, 12, 18, 24 and 48 hours post-infection. Larvae of *A. suum* were recovered from the gut contents 3 hours after inoculation of the eggs and by 6 hours most larvae were found in the caecum and colon and none were found in the gut contents by 18 hours post-infection. This experimental result shows that larval *A. suum*, and perhaps those of *A. lumbricoides*, penetrate the mucosa of the large rather than the small intestine and reach the liver from that site (Murrell *et al.*, 1997). A similar experiment but with different results from that carried out on pigs (Murrell *et al.*, 1997) was performed by McCraw (1975) who gave domesticated calves a single oral inoculum of 2 million eggs of *A. suum* and then carried out a post-mortem examination of one calf at 18 hours, 2, 3 (twice), 5, 7, 9, 11 and 13 days after infection. In this case, larvae were first recovered from the abomasal and duodenal walls and not from the liver until 5 days after infection. White patches (milk spot) were observed on the livers after 3 days. This study had been prompted by the fact that calves had been found with pneumonitis ascribed to migrating larvae of *A. suum* by McCraw and Lautenslager (1971).

In the pig experiments, larvae were recovered from the liver at 6 hours and by 48 hours some 600 larvae were recovered per gram of liver (Figure 9). A recovery rate of 600 larvae per gram of pig liver is extremely high in relation

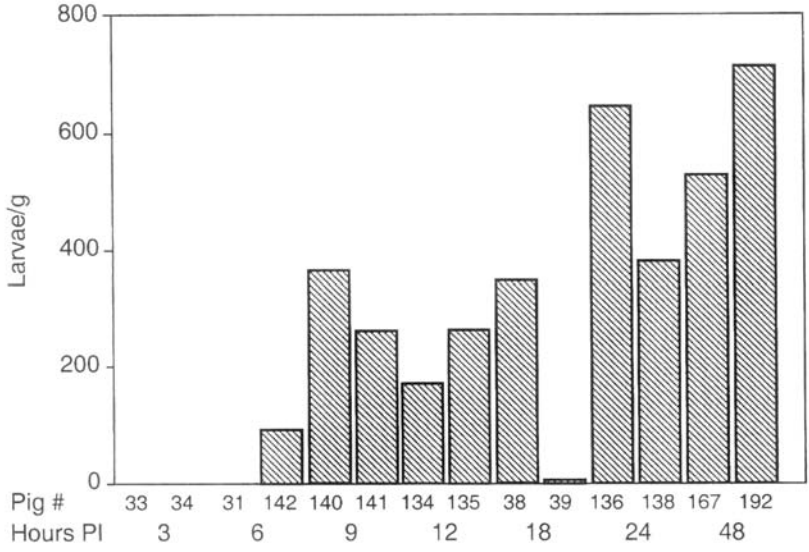

Figure 9 The number of *Ascaris suum* larvae recovered per gram of pig liver following oral doses of infective eggs. (Reproduced from Murrell *et al.*, 1997.)

to the number of eggs given to the pigs at the start of the infection (Murrell *et al.*, 1997). There are obviously extremely heavy losses before the *A. suum* reach maturity in the pig's small intestine. If the liver of a full grown pig varies from 1.5 to 2.0 kg in fresh weight (Getty, 1975), the liver of a 25 kg pig might weigh around 0.5 kg and so be carrying 300 000 larvae under the conditions employed by Murrell *et al.*

The finding that so many of the larvae reached the liver also raises questions about the degree of liver pathology caused in humans, assuming significant numbers of *A. lumbricoides* larvae reach the liver at any one time. Caution must be exercised when considering the results obtained from calves and pigs. These animals experienced special experimental conditions designed to approach a research question; it is unlikely that any human would ever ingest so many eggs simultaneously under natural conditions. There is, however, the notorious case in which the food at a festive meal was spiked with massive numbers of eggs of *A. suum* leading to acute illness in four male students (Phills *et al.*, 1972). The victims of this bizarre exposure reported to hospital about 14, 10, 14 and 14 days after the eggs would have been ingested and in each case pulmonary infiltrates and respiratory disturbance were the symptoms of concern. The larvae must have passed through the liver but there were no reports of liver malfunction or obvious illness associated with that phase of the parasite's tissue migration.

4.3. Transmission

The mode of transmission of *A. lumbricoides* depends under natural conditions on the ingestion of eggs containing infective larvae while human contact with these eggs depends on their degree of accessibility in a contaminated environment (Crompton, 1989). There is little new information to discuss on this aspect of transmission.

Since *A. lumbricoides* has a migratory phase during its association with humans there is the potential for vertical transmission to occur from a recently infected mother either transplacentally to the foetus *in utero* or during breast feeding to the suckling infant. Furthermore, it is well known that larvae of the ascarid *Toxocara canis* infect foetal pups *in utero* in dogs (Loke, 1982). Three observations suggest that *A. lumbricoides* may infect the human foetus or neonate. Chu *et al.* (1972) in Taiwan reported that a male infant, delivered after 8 months gestation by caesarean section, passed an adult *A. lumbricoides* at 2 and 4 days after delivery. Later after the delivery, ten adult *A. lumbricoides* were found on the maternal side of the placenta. Rathi *et al.* (1981) described a case of intestinal obstruction in a 45-day-old infant in India; it is difficult to see how these worms could have been acquired and achieved such a size if infection had occurred after birth. More recently, da Costa-Macedo and Rey (1990) detected fertile eggs of *A. lumbricoides* in the stools of a 40-day-old infant in Brazil and eggs were no longer found following treatment with levamisole. Against the context of the number of ascariasis cases that occur annually and globally, the occurrence of vertical transmission would appear to be extremely rare. What is more interesting is the implication that the migration of larval *A. lumbricoides* from the intestine to the liver and lungs is not a random process. Had the event been random then many more cases might have been expected of finding patent infections in young infants.

4.4. *Ascaris lumbricoides, A. suum,* Humans and Pigs

People, pigs and roundworms have lived together for several millennia especially in rural areas where generations of people have supplemented the fruits of subsistence farming with pork. Although *A. lumbricoides* L. 1758 and *A. suum* Goeze 1782 continue to be discussed as if they are separate species there are major difficulties in distinguishing them (Crompton, 1989). Cross-infectivity can undoubtedly occur and this process, coupled with a technique developed by Jungersen *et al.* (1997), means that it should now be possible to settle the matter of whether *A. lumbricoides* and *A. suum* are the same or separate species. Worms of one sex originating from worms retrieved at an appropriate time from one host can be transferred to pigs and worms of the other sex retrieved from the other host could also be transferred to the pigs. The

reciprocal transfers could also be carried out. The result of this hybridization experiment would require the production of eggs which give rise to successive generations of fertile worms thereby establishing in biological terms that *A. lumbricoides* and *A. suum* are the same species (Cain, 1954). This type of study is now in progress (Jungersen *et al.*, 1996) and the outcome is awaited with great interest.

Hawley and colleagues (Hawley and Peanasky, 1992; Hawley *et al.*, 1994) have reinvestigated the long established fact that *A. lumbricoides* and *A. suum* contain inhibitors of host trypsins (Chandler and Read, 1961) with reference to host specificity and cross-infectivity. Hawley and Peanasky (1992) prepared inhibitors of trypsin from *Ascaris* from pigs and also from *Ascaris* from humans. By means of polyclonal antibodies they showed that the trypsin inhibitors have similar epitopes but on measuring equilibrium constants they found that the trypsin inhibitor from pig *Ascaris* formed weak complexes with trypsin prepared from humans. Human *Ascaris* formed strong complexes with trypsin prepared from either host. A further experiment involved immersing live *Ascaris* from pigs in physiological saline containing either porcine trypsin or human trypsin. After five days, the *Ascaris* were still alive after exposure to porcine trypsin but dead and digested in the presence of human trypsin. Hawley *et al.* (1994) reviewed the proteinase inhibitors detected in extracts of *A. lumbricoides* and *A. suum*; these include substances which inactivate to different degrees mammalian carboxypeptidase, chymotrypsin, elastase, pepsin and trypsin. In the case of *A. suum*, the proteinase inhibitors are synthesized by the parasite and can inactivate serine and aspartic acid proteinases and a metallopeptidase. Further work indicated that in whole worms, as opposed to worm extracts, the inhibitors act *in situ* rather than as a secretion into the host's intestine.

The fascinating point from the work reviewed by Hawley *et al.* (1994) is the speculation that *A. lumbricoides* can survive in the intestines of humans and pigs because it inhibits trypsin of either source whereas *A. suum* cannot survive in humans because its trypsin inhibitor is ineffective in the human intestine. This explanation may prove to be too simple because cross-infectivity undoubtedly occurs (Lysek, 1967; Crompton, 1989; Grove, 1990). If host specificity is based on proteinase inhibitors then there is no difficulty in explaining how *Ascaris* of human origin can become established in pigs. It is less easy to explain the successful establishment of *Ascaris* from pigs in humans. Takata (1951) carried out a courageous experiment and set up patent infections of *Ascaris* from pigs in human volunteers but there were significant differences in the course of the infection when compared with *Ascaris* from humans, particularly in the prepatent period. This seems unlikely to be attributable to the worm's proteinase inhibitor activity.

Anderson *et al.* (1993), Anderson (1995) and Anderson and Jaenike (1997) studied humans and pigs from rural Guatemala and other locations to see

whether there were two distinct strains or species of the parasite and to assess the amount of cross-infectivity. The approach, based on a comparative analysis of ribosomal DNA of worms obtained from people and pigs, was extended to study the source of *Ascaris* infections in people from North America. The key results from this body of elegant research are: (1) an *Hae III* restriction site distinguishes two classes of rDNA repeats with >96% of the pig *Ascaris* showing the site and <2% of the human *Ascaris* from regions of endemic ascariasis; (2) the cases of apparently natural ascariasis in North America result from infection with *Ascaris* from pigs; (3) there is little evidence of natural cross-infectivity amongst humans, pigs and the two species (?) of *Ascaris* when all four occur naturally in the same community. Anderson and Jaenike then concluded that programmes aiming to control *Ascaris* infection in a human population could safely ignore zoonotic infections from pigs. Recent results from a genetic analysis of *Ascaris* obtained from people and pigs living in a long established rural community in China indicated that the worms represented two reproductively isolated populations (Weidong Peng *et al.*, 1998). Evidence for reproductive isolation came particularly from ribosomal DNA analysis and so may provide epidemiologists with markers to use in studies of cross-infectivity.

The view that relatively few infections of pig *Ascaris* are currently occurring in humans where ascariasis is endemic is an important result because control programmes based on chemotherapy for *A. lumbricoides* would be futile if free-range pigs constantly required treatment for *A. suum* (Peng *et al.*, 1996). Yadav and Tandon (1989) suggested the pigs could serve as a reservoir host for *Ascaris* infections in humans but Talapa and Bobyleva (1982), after an epidemiological survey of villagers living and working amongst pigs, found relatively little evidence of cross-infectivity. Out of 805 villagers working with 25 000 pigs, only 32 had ascariasis and yet manure used by the villagers on their crops contained viable eggs of *Ascaris* from pigs. In a similar community not connected with pig farming, the prevalence of ascariasis in the people was found to be very similar.

Molecular analysis of *Ascaris* DNA might help to resolve the issue as to whether *A. lumbricoides* and *A. suum* are the same or separate species. Xingquan *et al.* (1999) compared sequences of nuclear ribosomal DNA regions from worms obtained by chemotherapy from people in Australia, Bangladesh and China with worms obtained at post-mortem examination from pigs in Australia, Denmark, Scotland and the USA. Some of the results did not help to resolve the issue but six nucleotide differences between the two groups of *Ascaris* were detected in one of the sequences. These differences were detected regardless of the geographical origins of the samples and so would tend to support the notion that *A. lumbricoides* and *A. suum* are separate species. However, Xingquan *et al.* emphasize that reciprocal cross-mating trials (Jungersen *et al.*, 1996) need to be carried out to reach a firm conclusion.

4.5. Diagnosis

The detection of *Ascaris* eggs in fresh or fixed stool samples examined by bright field microscopy remains the most reliable means of identifying cases of ascariasis in both individual patients or community surveys. There is still no convenient method for discriminating between an egg of *A. lumbricoides* or *A. suum*; we assume that the eggs are those of *A. lumbricoides* if the host is human. While this assumption may be safely made, our inability to distinguish these eggs under the microscope hampers environmental studies where infected people and infected pigs live together (Schulz and Kroeger, 1992).

Efforts have been made to develop new diagnostic methods since Ransom introduced stool examination in 1856. Generally, prospects for serodiagnosis in human hosts are considered unpromising due to cross-reactivity (Wakelin *et al.*, 1993). A detailed attempt has been made in Denmark to compare an ELISA test and a histamine release test to detect *Ascaris* infections in pigs (Bogh *et al.*, 1994). Pigs used in the investigation were followed from farm to slaughter house and their infection status was carefully defined based on the numbers of intestinal worms, the numbers of eggs in stools and the numbers of liver milk spots. The most sensitive results were obtained with an ELISA based on larval ES antigen which accurately identified infected pigs with as few as three milk spots. This procedure may become useful in a developed country where sophisticated meat production is a major industry but spin-offs for developing countries with endemic ascariasis seem remote at this stage. Hall and Romanova (1990) also explored a novel method of diagnosis based on analysing human urine with gas liquid chromatography for metabolites excreted by *A. lumbricoides*, principally 2-methyl butyramide and 2-methyl valeramide. The technique proved to be successful and quantitative in that the amounts of metabolite detected correlated significantly with the worm burdens of the hosts. It is difficult to see how this technique can be adapted for community use in the near future but urine is regularly collected in surveys and can be examined quickly and cheaply with reagent strips for such infections as schistosomiasis haematobium (Montresor *et al.*, 1998).

In hospitals in industrialized societies or urban settings techniques such as ultrasound (Cremlin, 1982), radiography (Choudhuri *et al.*, 1986) and endoscopy (Khan *et al.*, 1993) are available for the diagnosis of ascariasis in individual patients. These procedures are not useful for the planning and managing of community control programmes.

5. GEOGRAPHICAL DISTRIBUTION

5.1. Historical Record

Most helminthological texts include a section to show that soil-transmitted nematodes have been infecting humans for a few thousand years. For example, eggs of a nematode identified as *Ascaris lumbricoides* have been found in a cave in Tennessee inhabited by humans 2177 ± 145 years ago (Faulkner *et al.*, 1989). And the most recent and fascinating addition to the list will be the inclusion of a species of *Trichuris* (presumably *T. trichiura*) found in scrapings from the damaged rectum of the ice mummy from Hauslabjoch by Aspöck *et al.* (1995). This mummy, recovered from melting ice at an altitude of 3516 m in the Ötztaler Alps, has proved to be the body of a young (by modern standards) man who lived over 5000 years ago. The presence of eggs in one individual is not particularly surprising and neither is the mummified evidence of infections of *Ancylostoma duodenale, Ascaris lumbricoides* (or *suum?*), *Enterobius vermicularis, Schistosoma haematobium, S. japonicum, Taenia* sp., *Trichinella spiralis* and *T. trichuris* from ancient communities around the world (Cockburn and Cockburn, 1980). Of much greater interest are the detailed writings which indicate that our ancestors were concerned to relieve themselves of the infections. Our ancestors may have had no conception of infection and transmission (Grove, 1990) but they undoubtedly took detailed care to rid themselves of worms.

The invention of writing probably occurred about 5500 years ago, around the time when the ice man was alive (Young, 1971). From this time on the Egyptians and Chinese began to record observations about worms and prescriptions for expulsion. For example, traditional Chinese medicine can be traced back for thousands of years (Zhang and Wu, 1991) with numerous recipes for dealing with intestinal complaints (Ou, 1989). The implication from all these ancient writings is that since settled communities arose the inhabitants have been concerned to be free from intestinal nematode infections.

5.2. Current Prevalence and Demography

The overall global estimate of 1.4 billion people currently harbouring *A. lumbricoides* (WHO, 1996b; Bundy *et al.*, 1990) is difficult to appreciate and is so often quoted that it loses its impact even on experienced clinicians and helminthologists. Similarly, although we may record that ascariasis occurs in over 150 of the world's 218 states and territories, at least that was the situation before the break up of Yugoslavia (Janssens, 1985; Stürchler, 1988; Crompton, 1989), such statistics do little more than draw attention to the existence of a

widespread infection. Prevalence estimates between countries, however, are found to be markedly different as are prevalence values for communities within the same country. For example, the average prevalence of ascariasis in Kenya was recently estimated to be about 38% and that in the Ivory Coast at about 17% (see sources cited in Crompton, 1989). How such differences are to be explained is not obvious, certainly if national profiles are compared (Table 1). Both are coastal countries in the central zone of Africa, both have experienced European colonialism and both are classified by UNICEF (1998) as developing countries rather than members of the even more impoverished group of least developing countries.

Perhaps the apparent differences between the estimated prevalence for ascariasis in the example of Kenya and the Ivory Coast stem from the fact that the available data are inadequate because they are derived from deficient sampling procedures and poor application of diagnostic techniques. Many years will pass before that criticism can be refuted with confidence but the results from three recent attempts to estimate the overall prevalence of ascariasis in the People's Republic of China are important for strengthening confidence in prevalence values because they show that different groups of researchers working independently, with different methods and with different levels of access to information can achieve remarkably close agreement. Yu Senhai (1992) concluded that the number of cases of ascariasis in China ranged from 523–539 million, Chan *et al.* (1994a) estimated the figure to be 568 million and Peng *et al.* (1995, 1998a) considered the figure to be 532 million; in each case the percentage prevalence value for PR China would be very similar provided that the same figure for the national population was used.

Anderson (see Crompton *et al.*, 1989) expressed the view that prevalence cannot in any sense be a useful score when examining an infectious agent such as *A. lumbricoides*. That opinion holds when seeking to understand the population biology and epidemiology of the parasite (see Section 6) but for many public health workers with the facilities available to them in developing countries, prevalence, based on the straightforward microscopical identification of helminth eggs in stool samples, remains one measurement that can be made. Recognizing this and noting that the clinical aspects of ascariasis are difficult to detect, Chan *et al.* (1994b) developed a system for using prevalence data to estimate morbidity levels (see Section 2).

Prevalence values vary between communities in a country, for example from 98.2% to 0.9% in Nigerian communities (Crompton and Tulley, 1987) and from 76% to 0% in relatively adjacent villages in Ghana (Annan *et al.*, 1986). In the study in Ghana, the same team of workers and methods were used and the differences cannot be explained by technical errors. Within a country, knowledge of prevalence of ascariasis and its distribution is particularly important for the design and management of control programmes. Consideration of the distribution of prevalence is crucial in the decision about where control

programmes should be located, about the numbers of drug doses needed and how to optimize delivery.

5.2.1. Ascaris *Infection in the United Kingdom*

The earliest recorded case of ascariasis in the UK appears to be the man who became known as 'Pete Marsh'. He was murdered some 2500 years ago and his corpse, containing worms identified as a species of *Ascaris* and a species of *Trichuris*, turned up in a bog in Cheshire (see Owen, 1986). Then as the population of England began to expand, urban settlements lacking adequate sanitation developed in mediaeval times and bizarre ideas about medicine prevailed (Rawcliffe, 1995). Ideal conditions for *A. lumbricoides* were established and the parasite thrived in England (Tyson, 1683).

Figures published by Owen (1986) indicated that on average 1015 cases of *A. lumbricoides* were being reported annually in Britain on the basis of data in the weekly Communicable Disease Reports. There is no means of knowing whether some or all of these cases are infections with *A. suum* as noted recently by Anderson (1995) who investigated cases in N. America. Reports of *Ascaris* infections in recent years in Scotland are set out in Table 10. Clearly these data, in relation to a population of around five million, need not cause much public health concern but it is interesting to note that 45 of the reported cases were found in adults (aged 18 to 67 years; average 31) and that 18 cases of infection (34%) were judged to be related to the travel experience of the patients (Table 10). The distribution of the reports can also be related to the various regions of Scotland. Eighteen and 15 of the reports came from Lothian and Grampian regions respectively, both in the Eastern half of the country. These regions also accounted for 12 of the travel-related reports. Perhaps the travel-related cases

Table 10 Reports of *Ascaris* infections in Scotland, 1993–1997

Year	Total	Males	Females	Unknown[a]	Adults	Children	Travel-related
1993	13	4	7	2	11	2	2
1994	12	2	5	5	10	1	6
1995	13	6	6	1	10	2	6
1996	6	3	3	–	6	–	3
1997	9	5	4	–	5	4	1
Totals	53	20	25	8	42	9	18

Data abstracted from the laboratory reports of regional Health Boards to SCIEH (Scottish Centre for Infection and Environmental Health).

[a] Sometimes the sex and/or age of a patient is not recorded.

involved *A. lumbricoides* and the remainder mainly *A. suum* connected with pig husbandry unless infective eggs of *A. lumbricoides* had reached Scotland on imported fruits and vegetables as has occurred in Finland (Raisanen *et al.*, 1985).

5.3. Urbanization

Urbanization in the context of developing countries results from the unplanned, uncontrolled and constant migration of people from the traditional rural environment to periurban slums, shanty town and squatter settlements. In the main, this type of migration proves to be irreversible as poorly educated, unskilled people flow to the cities in search of work. The countryside they leave is then depleted of the labour force required to grow crops for national nutritional needs and export (Jardel, 1991; Crompton and Savioli, 1993). At the present time, the annual growth rate of the urban population in sub-Saharan African countries is 5.0% compared with 0.8% for industrialized countries (UNICEF, 1998). There are also found in the large cities of developing countries long established slums whose residents were born there and now constitute a group living on the margins of economic, social and political activity. The urban poor, through neither their own fault nor that of city authorities, find themselves trying to survive against overstretched services for water supply, sanitation, garbage disposal and health care. In 1990, the International Centre for Diarrhoeal Disease Research (ICDDR) reported that of the 7 million inhabitants of Dhaka, Bangladesh, about 3.5 million were living in slums, 6% of these had access to primary education and 3% to primary health care (ICDDR, 1990).

The social and environmental conditions in the unplanned slums of developing countries are ideal for the persistence of *A. lumbricoides* and *T. trichiura*, both of which depend on the faeco-oral route for transmission (Table 11). Surveys undertaken in Hyderabad (India), Kuala Lumpur (Malaysia), Coatzacoalcos (Mexico), Lagos (Nigeria), Manila (Philippines) and Freetown (Sierra Leone) to determine urban prevalences of *A. lumbricoides* found values of 35%, 64%, 55%, 68%, 80% and 43% respectively (Reddy *et al.*, 1986; Chia Wee Yan, 1978; Forrester *et al.*, 1988; Fagbenro-Beyioku and Oyerinde, 1987; Auer, 1990; Webster, 1990). In theory, if resources become available, the population density in urban slums should facilitate drug delivery and opportunities for health education. Individuals also have access to purchasing anthelminthic drugs for treatment of their families; unfortunately this opportunity is often undermined by the counterfeit drug problem (Section 8.2).

All authorities agree that the proper use of functional, culturally appropriate sanitation is the key to permanent relief from ascarasis and many other much more severe infections. Crompton and Savioli (1993) addressed this problem by reference to ascariasis in the Lagos conurbation (Table 11).

Table 11 Factors influencing the persistence of *Ascaris lumbricoides* in urban communities in developing countries

Estimates for 1990[a]	
Population	2500 million
Daily faecal output of population	500 000 tonnes
Number infected with *A. lumbricoides*	1000 million
Daily faecal output contaminated with eggs of *A. lumbricoides*	200 000 tonnes
Daily discharge of eggs of *A. lumbricoides*	2×10^{14} eggs
Population of urban communities	750 million
Daily faecal output of urban population	150 000 tonnes
Number of urban people infected with *A. lumbricoides*	300 million
Daily faecal output contaminated with eggs of *A. lumbricoides* in urban communities	60 000 tonnes
Daily discharge of eggs of *A. lumbricoides*	6×10^{13} eggs
Estimates for urban communities in the year 2000[a, b]	
Population	2200 million
Daily faecal output	440 000 tonnes
Number infected with *A. lumbricoides*	880 million
Daily faecal output contaminated with eggs of *A. lumbricoides* tonnes	1 760 000
Daily discharge of eggs of *A. lumbricoides*	1.76×10^{14} eggs
Estimates for the conurbation of Lagos, Nigeria, in 1991[b, c]	
Population	5.7 million
Number of slum and shanty dwellers	2.85 million
Daily faecal output of poor people	570 tonnes
Daily faecal output contaminated with eggs of *A. lumbricoides*	2.9×10^{11} eggs

[a] 1. World's population in 1990 was about 5000 million with about half living in developing countries.
2. Assumed that about 1000 million people in developing countries are infected with *A. lumbricoides*.
3. About 200 g stool produced per person daily.
4. About 1000 eggs of *A. lumbricoides* present daily in each gram of stool from an infected person.

[b] Assumed that the prevalence of *A. lumbricoides* will not have changed and that overall the proportion of infected people in rural and urban communities will be the same.

[c] 1. Based on the results of the Nigerian 1991 census.
2. Perhaps as many as 50% of the population of Lagos may constitute the urban poor.
3. Various estimates suggest that >50% of the population of Lagos may be infected with *A. lumbricoides*.

(Reproduced from Crompton and Savioli, 1993).

At some time the now industrialized countries faced this situation and overcame it as economic growth emerged over a period of 200 or 300 years. That seems a grim prospect for the populations of sub-Saharan Africa. Scientists are charged with adding to natural science; politicians must decide how to make best use of this knowledge for the majority of the people they serve. That comment is of course not directed at the Nigerian or any other government.

6. POPULATION BIOLOGY

6.1. General Features

Ascaris lumbricoides is an excellent example of a helminth displaying all the criteria of a macroparasite with a direct life cycle (Anderson and May, 1991). The mathematical framework developed by these authors has established a much clearer understanding of the results of many epidemiological surveys which have described the distribution and abundance of *A. lumbricoides* and other soil-transmitted nematodes. As a result of the Anderson and May framework there is universal recognition that the intensity of infection is the key variable to be studied if the transmission, parasite population regulation and morbidity are to be explained and manipulated to the benefit of individuals and communities. When sample sizes are adequate and cross-sectional surveys are carried out in areas where ascariasis is endemic, the data can be arranged to reveal three patterns. First, prevalence rises rapidly once infancy has passed and tends to remain high. Secondly, intensity rises rapidly and peaks during childhood before declining steadily. Thirdly, the frequency distribution of numbers of worms per host is observed to be overdispersed. Numerous examples of these patterns have been collated and examined by Anderson and May (1991).

The persistence of *A. lumbricoides* in a community and its contribution to the regulation of its population in that community depend on the numbers of eggs that are produced, the numbers that embryonate in the environment and attain infectivity and the number of these which are accessible to susceptible hosts. The special significance of the intensity of infection for a dioecious macroparasite like *A. lumbricoides* is that the composition of the infra-population of worms in a host will determine whether insemination takes place and whether density-dependent constraints on female worm fecundity apply. Recent experimental work with pigs and *A. suum* has revealed new features of the reproductive biology of that parasite (Jungersen *et al.*, 1997). In these experiments, adult female *A. suum* were transferred orally to pigs known to have had no previous experience of the parasite and worm egg production was followed by faecal examination. Hardly any eggs, and those were unfertilized and lacking the characteristic sticky coat, were detected two weeks after transfer. However, when male *A. suum* were transferred orally to the same pigs 28 days after the females, normal egg production was resumed. Related studies have been carried out over the years with the acanthocephalan *Moniliformis moniliformis*, another dioecious macroparasite which attains sexual maturity in the small intestine of rats (Crompton, 1985). The fecundity of a female *M. moniliformis* depends on the structure of the infra-population, the duration of the male–female contact and the quality of the host's diet;

apparently similar factors influence the fecundity of *A. suum* and probably of *A. lumbricoides*.

All the processes which generate the population of infective eggs of *A. lumbricoides* that are accessible to susceptible hosts determine the parasite's basic reproductive rate. Anderson and May (1991) have defined this for a parasite such as *A. lumbricoides* as the number of female offspring produced during the life span of a mature parasite that survive to maturity in the absence of density-dependent constraints on population growth. Unless the reproductive rate achieves a value of unity or better, the parasite will perish. During the course of its evolution, *A. lumbricoides* has acquired a prodigious fecundity rate with female worms producing some 200 000 eggs per day for about a year (Crompton, 1989). There is either a rapid rate of loss of infectivity of many of the eggs, perhaps due to many being exposed to sunlight, or a rapid decline in the time for which they are accessible to human hosts. Under experimental conditions, Krasnosos (1978) showed that some embryonated eggs of *A. lumbricoides* were still viable after 14 years but that may have little bearing on natural events in human comments. Human behaviours such as geophagy (Wong *et al.*, 1991; Geissler *et al.*, 1998), the widespread use of untreated night soil as a fertilizer for crops (Kilama, 1989) and the properties and structure of the soil (Mizgajska, 1993) are crucial factors in enabling the reproductive rate of *A. lumbricoides* to remain secure. Although there would still seem to be little chance of a control programme being able to disrupt transmission to the extent that the reproductive rate falls below 1 (Warren, 1982), unless appropriate sanitation is installed and used properly, health providers and planners should consider options for strengthening control activities in addition to the distribution of anthelminthic drugs (Section 7). The more thorough the knowledge of conditions prevailing where control measures are needed, the better the prospects for achieving the intended control targets.

6.2. Predisposition

The notion that individuals in a community are in some way predisposed to harbour relatively constant intensities of infection with *A. lumbricoides* is most intriguing. Parasitologists generally identify predisposition by following the course of reinfection in individuals after chemotherapy. Studies in India (Haswell-Elkins *et al.*, 1987), Myanmar (Thein Hlaing *et al.*, 1987), Nigeria (Holland *et al.*, 1989), Mexico (Forrester *et al.*, 1990), Malaysia (Chan *et al.*, 1992) and Thailand (Upatham *et al.*, 1992) have found that infection intensities attained in individuals on reinfection after anthelminthic chemotherapy correlated positively with pre-treatment intensities in the case of *A. lumbricoides* and other species of soil-transmitted nematode.

Holland *et al.* (1989) found that the relationship between primary schoolchildren and their worm burdens continued after three rounds of expulsion chemotherapy with levamisole. On the basis of the numbers of *A. lumbricoides* passed by the treated children, worm burdens could be classified into heavy, light and zero. Fifty-one per cent of the children judged to be infected at the start remained in that state and 31% of those in the heavy category at the start were consistently found in that category. Results like these are interpreted as evidence of predisposition to a given worm burden but three points should be noted. First, some individuals remain uninfected despite exposure to infective stages. How such people achieve this state merits future study, particularly since researchers do not present any evidence of the use of medication or sanitation over and above what is available in the community. Secondly, in the Nigerian study (Holland *et al.*, 1989) exposure to infection with *A. lumbricoides* in terms of numbers of eggs in soil samples was the same regardless of the intensity status of the subjects. Thirdly, the evidence for predisposition actually exists at the population level and is demonstrated by statistical investigation of the results. In all the studies cited above, some individuals change infection status during periods of reinfection.

Results obtained recently in rural China by Peng *et al.* (1998b) provide further evidence for some degree of predisposition to intensity of infection with *A. lumbricoides*, this time without the use of expulsion chemotherapy. In a longitudinal study, stool samples were collected at regular intervals and egg

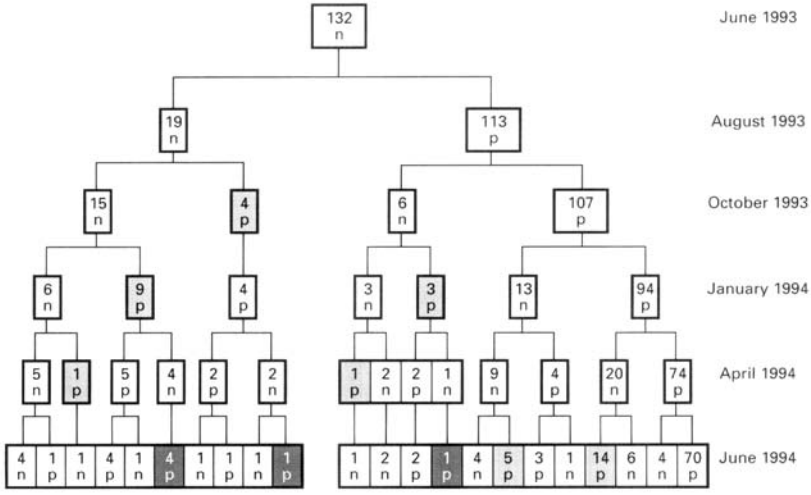

Figure 10 Transmission dynamics of *Ascaris lumbricoides* in the 132 cases who were found to be infected at the first survey. p, positive, infected; n, negative, uninfected. Boxes shaded with sparse dots indicate first reinfection, and boxes shaded with dense dots indicate second reinfection. (Reproduced from Peng *et al.*, 1998b.)

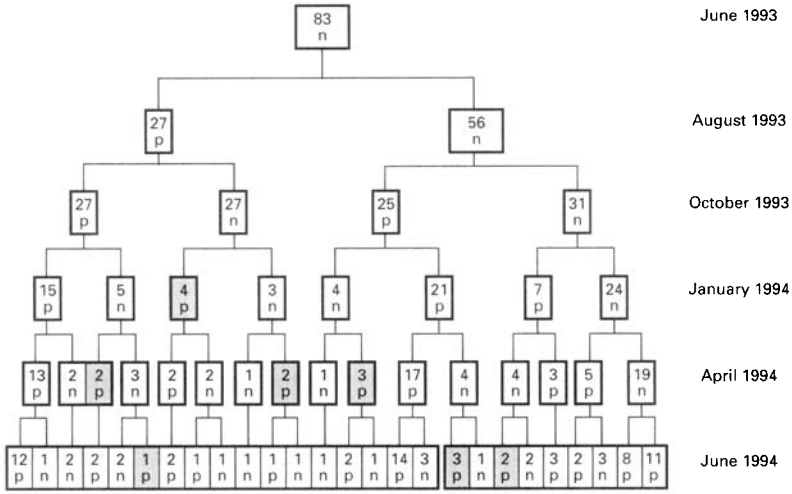

Figure 11 Transmission dynamics of *Ascaris lumbricoides* in the 83 people who were found to be uninfected at the first survey. p, positive, infected; n, negative, uninfected. Boxes shaded with dense dots indicate second infection. (Reproduced from Peng *et al.*, 1998b.)

counts were recorded for a cohort of 215 people who received no treatment over a year. The infection status of 132 people found to be infected at the time of the first sample is shown in Figure 10 and that of 83 people found to be uninfected is shown in Figure 11. Overall, there was a tendency for the initial infection intensity in the two groups to remain unchanged indicating the stability of the host–parasite relationship. Predisposition was again evident; analyses based on either the whole cohort of 215 or on the two groups revealed that those with initial light infections tended to retain light infections and those with initial heavy infections tended to harbour infections. Since the life span of *A. lumbricoides* is reckoned to be a year and the prepatent period to be a few weeks (Crompton, 1989), the apparent predisposition in this community must have resulted from natural events related to the degree of susceptibility of the hosts to the parasite. Peng *et al.* (1996) had already shown that infective eggs are widely distributed in the soil of the community and that night soil is used as a traditional fertilizer. Furthermore, a study by Ibrahim *et al.* (1994) suggests that the *A. lumbricoides* in a given population breed at random in a manner that would not favour some worms having progeny that are more infective than those of other worms.

Given that the evidence for predisposition to intensity of infection with *A. lumbricoides* is based on sound study designs and appropriate methods of data investigation, researchers are faced with the challenge of explaining how

predisposition occurs. Are the individuals in the tail of the probability distribution of worm numbers per person found there not by chance but as a result of social, behavioural, genetic or nutritional factors (Anderson and May, 1991)?

Palmer *et al.* (1995) investigated the possible role of host immune responses as a determinant of predisposition to intensity of *A. lumbricoides* infection in a large sample of Bangladeshi children. There is a vast body of evidence from the study of animal host–parasite relations to show that the genetic background of the hosts is a key factor in resistance to specific parasites (Wakelin, 1978). It is an entirely reasonable assumption that variations in the genetic backgrounds of these Bangladeshi children, expressed as variations in antibody responses, might explain their observed predisposition to *A. lumbricoides* infection. Accordingly, they sought to disprove the null hypothesis that there were no differences in the antibody responses to antigens of *A. lumbricoides* between persistently lightly infected children (18 worms per child) and persistently heavily infected children (110 worms per child). The study design was extremely rigorous; there were no significant differences between the two groups as regards nutritional status or polyparasitism which could have affected the results due to cross-reactivity interfering with the immunological tests. It seems unlikely that the degree of exposure of the children in the two groups to infective eggs of *A. lumbricoides* would account for the persistently different worm burdens (Holland *et al.*, 1989; Peng *et al.*, 1998b).

Palmer and colleagues prepared antigens from adult *A. lumbricoides* and from larvae obtained from embryonated eggs which had been maintained and stimulated to hatch *in vitro*. ELISA methods were used to measure isotype-specific responses and an IgE capture ELISA assay was used to measure total IgE levels. Overall, this is the most comprehensive study of the human immune response to *A. lumbricoides* carried out to date. However, despite all the careful technology, the sample sizes and the striking differences between the worm burdens to which the children had been shown to be consistently predisposed, Palmer *et al.* (1995) could not offer an immunological explanation for predisposition. They concluded that with this level of analytical sensitivity, antibody responses simply reflect the intensity of infection and may not play a significant role in protecting against heavy infections.

For ethical reasons, experiments cannot be carried out to study the population biology and observed predisposition of humans to infection intensity and status regarding *A. lumbricoides*. However, a comprehensive experimental programme has been carried out by Boes *et al.* (1998) to investigate the frequency distribution of numbers of *A. suum* in pigs infected either naturally (50 pigs in an *Ascaris*-egg contaminated field) or experimentally (38 pigs given 1000 *Ascaris* eggs twice weekly for 12 weeks). As with human hosts and *A. lumbricoides*, the pig–*A. suum* interactions resulted in the worm's being highly overdispersed in their hosts and the population dynamics of the *A. suum* in pigs closely matched that of *A. lumbricoides* in humans. Interestingly, on

reinfection following anthelminthic treatment pigs were found to be predisposed to high or low infection intensities; the results again mirror what has been found with humans and *A. lumbricoides* (Holland *et al.*, 1989). How a host acquires and responds to a worm burden requires experimentation and Boes *et al.* are to be encouraged to exploit the pig–*Ascaris* system with a view to explaining how humans acquire and respond to *A. lumbricoides.*

Much interest has been expressed in identifying individuals with a 'predisposition' to support high intensities of infection with *A. lumbricoides* and treating them preferentially in a control programme (Upatham *et al.*, 1992). In theory, this appears to be a wise strategy; individuals at risk of life-threatening aspects of ascariasis such as intestinal obstruction are protected; the output of eggs into the environment will be reduced significantly; drug costs will be reduced to a minimum. In practice, however, the costs of identifying the wormy individuals is considerable in terms of technical staff time and there is likely to be poor compliance and even hostility in a community where individuals known to be infected are not offered treatment (Asaolu *et al.*, 1991).

7. CONTROL OF ASCARIASIS

In 1993 the World Development Report ranked intestinal helminths as the prime cause of infectious disease in children aged 5 to 14 years in developing countries (World Bank, 1993). This judgement was based on a deeper understanding of the distribution and abundance of these parasites (Section 5) and clearer insight into the relation between intensity of infection and morbidity at both the community and individual level (Sections 6 and 2). The control of *Ascaris lumbricoides* can no longer be considered in isolation; strategies are required to control the morbidity due to the four common species of soil-transmitted nematode which invariably occur concurrently in the same community and individual. Warren (1989) proposed that morbidity caused by nearly all the major helminth infections of humans could be controlled by an integrated programme of treatment using three drugs, albendazole, invermectin and praziquantel. This proposal has not yet been put to the test due in part to uncertainties over how drugs or their metabolites might interact in the body.

7.1. Strategies for Sustainable Control Programmes

Strategies for the control of ascariasis and the morbidity caused by infections of other species of intestinal nematode have been developed over the years by the Asian Parasite Control Organization (APCO), the World Health

Organization (WHO) and many clinicians, public health workers, nutritionists and parasitologists. Accounts of the development of these strategies with much practical advice and shared experience of how to develop and implement control programmes have been published by WHO (1967, 1968, 1987, 1990, 1996a, b), APCO (1980–1994), Crompton *et al.* (1985, 1989), Pawlowski *et al* (1991), Savioli *et al.* (1992), Bundy and Guyatt (1995), Bundy and de Silva (1998) and Albonico *et al.* (1998). While recognizing the importance of widespread access to culturally acceptable sanitation and the essential support of health education, control strategies currently depend on the application of modern anthelminthic drugs in the community in the light of our improved understanding of the population biology of the helminths (Anderson and May, 1991).

7.2. Chemotherapy

The World Health Organisation recommends that four anthelminthic drugs should be used in programmes designed to reduce morbidity due to *A. lumbricoides* and other intestinal nematode infections (WHO, 1995). Details of these drugs are set out in Table 12. The success of either a programme to control nematode-induced morbidity using chemotherapy or the treatment of an infected individual depends on the quality of the available drug (Table 12).

Table 12 Information about the therapeutic activity of anthelminthic drugs recommended by WHO

Drugs	Therapeutic activity against:				
	Ascaris lumbricoides	Hookworms (*Ancylostoma duodenale* and *Necator americanus*)	*Trichuris trichiura*	*Strongyloides stercoralis*	*Enterobius vermicularis*
Albendazole	4	3	2–3	2–3	4
Levamisole	4	2–3	2	2	2
Mebendazole	4	2–3	2–3	2	3–4
Pyrantel embonate	4	2–3	1	1	3–4

1. 1 = 0–19% cure rate; 2 = 20–59% cure rate; 3 = 60–89% cure rate; 4 = >90% cure rate.
2. Cure rate is defined as the proportion of treated individuals judged to be egg negative on one follow-up examination of a faecal sample.
3. Genuine comparative information is difficult to obtain depending on detailed knowledge of the drug and its dosage and the time of the follow-up examination.

(Based on information in WHO, 1996b)

Unfortunately, there now exists a sizeable counterfeit drug industry in which pharmaceutical products are sold and, on subsequent analysis, are found to contain less active ingredient than claimed, wrong ingredients, no ingredients or even toxic compounds. The counterfeit drug industry is a criminal activity (WHO, 1992) and public health workers and programme managers should consider not paying for drugs until quality is assured. To this end, the German Pharma Health Fund has sponsored research to develop simple thin-layer chromatographic procedures to enable drug quality to be checked in laboratories in developing countries (Pachaly, 1994).

Anderson (1989) proposed that such drugs should be used in the community on the basis of mass, targeted and selective treatment. Recently these usages have been modified to universal, targeted and selective treatment to avoid confusion over the use of the term mass treatment. The three forms of drug use are defined as follows (WHO, 1996b).

- Universal – population level application of anthelminthic drug in which the community is treated irrespective of age, sex, infection status or other social characteristics.
- Targeted – group level application of anthelminthic drug where the group may be defined by age, sex or other social characteristics irrespective of infection status.
- Selective – individual level application of anthelminthic drug where selection is based on diagnosis of current infection.

To date, there is no evidence to suggest that levamisole or pyrantel are experimental teratogens or embryotoxicants so these drugs would appear to be somewhat safer to use in women and infants than the benzimidazoles. However, as a general rule and unless well-trained medical personnel are available, the use of anthelminthic drugs is to be discouraged for women in the first trimester of pregnancy (see WHO, 1996a).

Asaolu *et al.* (1991) carried out a field trial in four rural Nigerian villages to compare the effects of mass (= universal), targeted and selective anthelminthic treatment with levamisole on infections with *A. lumbricoides*. The trial lasted a year, drug was given at 3-monthly intervals and the variable of interest was intensity of infection in each village community measured indirectly as egg counts by means of the Kato Katz technique. Details of the study and the main results are presented in Table 13. Mass or universal treatment proved to be popular and highly effective in terms of reducing the intensity of infection, targeted treatment was equally well received and was significantly effective but selective treatment proved to be problematic. In the village where selective treatment was applied (Table 13), the treated individuals undoubtedly benefited (epg fell from 30 839 ± 7710 to 801 ± 2024, $P<0.0001$) but there was no overall effect in the community. Furthermore, the residents of the village

Table 13 Community control of *Ascaris lumbricoides* in rural Nigeria: a comparison of mass, targeted and selective treatment with levamisole

Village	Iloba	Alakowe	Iyanfoworogi	Akeredolu
Population	523	334	445	595
Households	85	51	71	94
Mean (± SD) household size	6.1 ± 3.8	6.5 ± 4.3	6.2 ± 3.9	6.5 ± 4.4
Participation (%)	80	85.6	77.5	68.2
Treatment strategy	Control[a]	Selective[b]	Targeted[c]	Mass (Universal)[d]
Pre-treatment (epg ± SD)	7 542 ± 11 785	6 775 ± 10 790	9 057 ± 15 797	11 906 ± 17 219
Post-treatment (epg ± SD)	4 735 ± 8 137	4 259 ± 10 909	2 579 ± 6 529	1 489 ± 5 165
Statistical significance (pre v post)	P < 0.5	P < 0.1	P < 0.0001	P < 0.0001

[a] Treatment provided after the 'post-treatment' stool sample had been collected.
[b] Treatment provided for 36 individuals with more than 20 000 epg at the pre-treatment stool sample.
[c] Treatment provided for all children aged from 2–15 years.
[d] Treatment provided for all willing residents except for children under the age of 1 year and women known to be pregnant.

(Based on data in Asaolu *et al.*, 1991)

complained and questioned as to why so few of them were getting treatment when it was well known that many had worms. The most interesting result from this study was the finding from the targeted village where the intensity of infection fell in untreated residents aged 16 years or over. In this group (n = 94), the pre-treatment epg was 7742 ± 9782 and the post-treatment epg was found to be 4561 ± 8798 (P<0.0003). There was no evidence to indicate that this cohort of untreated, infected adults had received any form of anthelminthic prescription during the course of the trial; reduced exposure from infective eggs resulting from the targeted treatment of children appeared to be the best explanation (Asaolu *et al.*, 1991).

Much experience in terms of both operational research and community control programmes has now been gained from targeting anthelminthic treatment at school-age children infected with *A. lumbricoides* and other soil-transmitted nematodes. The procedure is to use one of the four recommended drugs according to the manufacturer's instructions with administration taking place through schools. The broader the spectrum of activity of the drug (Table 12), the greater the effect is likely to be but programme managers have to take into account availability of supply, drug quality, the prevalence and distribution of infections, community compliance and costs when the drug to be used is chosen.

Chan *et al.* (1994b) published a model to predict the effects of age-targeted anthelminthic chemotherapy in community control programmes designed to reduce morbidity by reducing intensity (Figure 12). They tested their model against data sets from control programmes in the Caribbean Island of

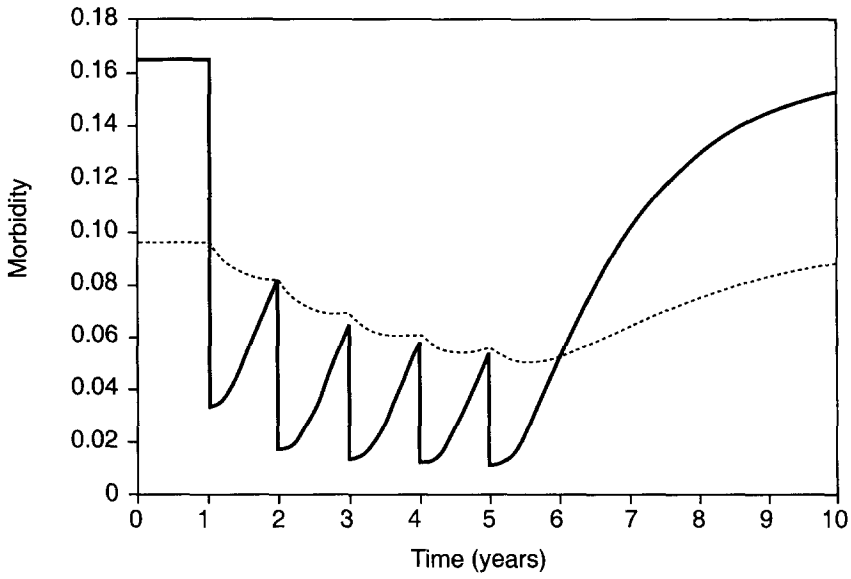

Figure 12 Predictions of the effects of age-targeted anthelminthic chemotherapy in a programme designed to reduce morbidity due to soil-transmitted nematodes. Child morbidity (———) and adult morbidity (– – – –) decline according to the model when the drug is given to 80% of the children and its efficacy is assumed to be 90% successful. Inevitably, morbidity rises in response to re-infection when drug administration is stopped. (Reproduced from Chan *et al.*, 1994b.)

Montserrat (Bundy *et al.*, 1990). The intensities of helminth infections in the untreated adult sections of both communities fell significantly as was also observed by Asaolu *et al.* (1991) in rural Nigeria. Holland *et al.* (1996a) extended the work in Nigeria by targeting levamisole at children under different treatment intervals (annually, 6-monthly and 4-monthly) to reduce the intensity of *A. lumbricoides* in village communities. In this study, the community in which the children had received three doses of levamisole was the one in which a statistically significant decline in intensity was detected in the untreated adults. In control programmed centres on chemotherapy the intervals between treatments must be calculated with care and then must be strictly adhered to. It can be difficult to sustain compliance once participants become aware of no longer being infected. The concept of reinfection by an agent that cannot be seen is difficult to explain and emphasizes the need for effective health education as part of a control programme.

In addition to the publications dealing with control measures in Montserrat, Myanmar and Nigeria mentioned above, the reader is referred to the results of studies in Japan (Yokogawa, 1985), South Korea (Seo, 1990), Pemba Island,

Tanzania (Renganathan *et al.*, 1995), Seychelles (Albonico *et al.*, 1996) and Sri Lanka (Sorensen *et al.*, 1996) and to the information reviewed by Albonico *et al.* (1998).

7.3. Health Education

A major theme of this review is to demonstrate that advances in our knowledge of the biology of *A. lumbricoides* and the epidemiology of ascariasis are being used to design strategies for the control of the morbidity caused by this nematode and its other soil-transmitted nematodes. Whether these strategies can be widely implemented and sustained in the long term in regions where ascariasis is endemic depends primarily on the response and efforts of recipient populations; will control programmes be able to continue without the financial input and technical resources of Western agencies? That question assumes that such control programmes will be integrated into the wider sphere of health provision offered through primary health care. Successful control activities have been integrated into other programmes such as those designed to improve nutrition and promote family planning (child spacing) (Yokogawa, 1985) or control schistosomiasis mansoni (Camillo-Coura, 1985) but these efforts have depended on considerable financial support.

Education intended to raise health awareness is one of the main elements of primary health care (Pawlowski, 1985). The challenge for those who have made the advances in knowledge is how to bring this knowledge into the experience of communities without clashing with their traditions and culture. Strategies for control programmes may be developed in Geneva and Washington but the tactics for sustainable implementation and compliance will depend on local communities. There is a strong case for seeking the guidance of anthropologists in addition to epidemiologists and clinicians as strategic planning evolves (Sanjur, 1982, 1989; Nations, 1985).

One example serves to support this view. Torlesse (1999) has recently investigated the contribution of infection with *Necator americanus* to iron-deficiency anaemia during pregnancy amongst the Temne people of Sierra Leone. Torlesse explored the traditional beliefs and practices of the Temne relating to soil-transmitted helminths and anaemia. Such matters cannot be studied in isolation from an ethnic group's total system of belief. The Temne recognize many supernatural influences; they distinguish between 'visible' and 'hidden' worlds. Ancestors, devils and witches inhabit the hidden world of the Temne and they are believed to have significant influence on fertility and the outcomes of pregnancy. There are also witches and others who inhabit the visible world of the Temne and they too are respected and feared for their influence on numerous facets of health and well-being. Introducing effective parasite control activities in communities such as the Temne in Sierra Leone

will first need to reassure the influential members of the visible world that their status could be strengthened rather than threatened by modern knowledge. That may not sit comfortably with the Temne's feeling for the supernatural; years of willing education will be required.

In general terms, there is abundant evidence to show that households with more education enjoy better health, both for adults and children. This finding holds true regardless of differences in research methodology, participating populations and time periods for many studies (World Bank, 1993). Maternal education, increasing and extending school enrolment especially for girls and all forms of continuing education can be expected to improve gradually the effectiveness of parasite control programmes. Evidence exists to support this claim. In a recent study in an urban slum area in Sri Lanka, de Silva *et al.* (1996) discovered that there was a highly significant correlation ($P = 0.004$) between the amount of education mothers had received and the prevalence of soil-transmitted helminth infections in their children. Presumably, regardless of living in an environment favouring the persistence of helminth infections, better educated women had better health awareness which served to protect their children from infection.

7.4. Sanitation

Permanent protection and long-term prevention and control of ascariasis and many other infections will not be achieved until the populations of countries where the infections are endemic have access to and understand how to use high quality sanitation. Anthelminthic drugs, no matter how skilfully they are delivered, cannot offer lasting relief because they cannot overcome environmental contamination with infective eggs. Sanitation and sewage treatment of the type routinely available in industrialized countries is far too expensive to be introduced in most developing countries (Crompton and Savioli, 1993). In 1990, the World Bank estimated that over 2 billion people lacked an adequate system for the safe disposal of their faeces. Ten per cent of the total burden of disease is attributable to infections which exploit the faeco-oral route of transmission (World Bank, 1993). The same two billion people invariably lack not only clean water but a regular supply sufficient to promote good hygiene practices. The nation-wide prevalence of ascariasis reached nearly 63% in Japan in 1949 as the country's infrastructure deteriorated during and after World War II. By 1982, nation-wide prevalence had fallen to around 0.05% due to the massive effort of the government and population to diagnose and treat cases (Yokogawa, 1985) and in response to the reconstruction of sanitation and sewerage underpinned by economic growth. Communities need not wait for the establishment of sanitation systems at a national level. In a longitudinal study involving monitoring the health and growth of 75 babies in St Lucia, Henry

(1981) found that in an area provided with water-seal latrines, not only did the occurrence of diarrhoea decline but also the prevalences of ascariasis and trichuriasis were reduced and childhood nutritional status improved in association with the improved sanitation.

Since every effort must be made to introduce affordable and culturally acceptable sanitation to support and sustain control programmes (Kilama, 1985), sanitary facilities must be properly sited and waste products treated to destroy the transmission stages of *A. lumbricoides* and other pathogens especially where 'night soil' is used routinely as a fertilizer for vegetables. Well tried methods are now available for extracting and quantifying ascarid eggs from a variety of soil types (Muller *et al.*, 1989; Mizgajska, 1993; Ajala and Asaolu, 1995). Schultz and Kroeger (1992) measured the number of *A. lumbricoides* eggs in soil samples taken from households in urban Brazil. The soil contamination data showed no significant correlation with the quality of latrines or even availability of flush toilets because these facilities were placed so that excreta seeped into the environment. Composting of excreta collected in pit latrines or the chemical treatment of sewage to destroy helminth eggs must be carried out before such material is used for fertilization of crops (Kilama, 1985; Ayres *et al.*, 1992).

The use of inadequately treated 'night soil' as a fertilizer represents a serious public health hazard even if horticultural productivity is improved. The uterine wall of the female *A. lumbricoides* deposits an uneven layer of sticky mucopolysaccharide on the outer surface of most eggs (Foor, 1967). These sticky eggs adhere to vegetables and, if they have not experienced adequate composting or sewage treatment to destroy the infective larvae, are then readily distributed in food markets. A survey of 20 types of vegetable sold in 40 Tokyo shops some years ago, at the time when Japan was tackling its ascariasis problem (Yokogawa, 1985), showed that eggs of *A. lumbricoides* were present on 1178 out of 2750 items examined (Kobayashi, 1980). Once the use of untreated waste water for crop irrigation was prohibited in Israel the national prevalence of ascariasis fell from 35% in 1948 to 1% in 1960 (Shuval *et al.*, 1985).

An interesting observation has been recorded by Penali *et al.* (1988) who were studying the parasites of five species of fish which are frequently eaten in the Ivory Coast. Unembryonated eggs of a species of *Ascaris*, assumed by the authors to be *A. lumbricoides*, were found in samples of gut contents of 27 fish from three of the five species examined. How the fish ingested these eggs is unclear but, whether from contaminated fish feed or from inadequately treated waste reaching the fish ponds, this finding further emphasizes the need for proper and safe disposal of waste materials in areas of endemic ascariasis. Perhaps this finding gives some credibility to the observation that 67% of those surveyed in a rural community in Malaysia thought that ascariasis was caused by eating fish (Chen, 1970).

Table 14 Estimates of the intensities of infection of soil-transmitted nematode infections (egp) in relation to the provision of facilities for plantation workers in Sri Lanka (after Sorensen *et al.*, 1994)

	Low country[a]			Up country[b]		
	Good[c]	Poor[d]	P[e]	Good[c]	Poor[d]	P[e]
Ascaris	7995	15 761	<0.001	12 806	13 528	0.41
Necator	194	308	<0.001	7	29	<0.001
Trichuris	425	93	<0.001	38	416	0.03

[a] Low country: rubber plantations, scattered housing.
[b] Up country: tea plantations, congested housing.
[c] Good: > 70% latrine coverage.
[d] Poor: < 20% latrine coverage.
[e] Kruskal-Wallis non-parametric test.

The provision of sanitation must meet certain conditions if it is to be effective in reducing the community burden of ascariasis. For example, in the plantations of Sri Lanka population density was shown to be as important as the provision of latrines (Table 14). Sorensen *et al.* (1994) concluded that housing and population density had to change in the plantation communities to achieve long-term relief from soil-transmitted nematodes regardless of the provision of latrines.

There is, however, no doubt that efficient sanitation and effective treatment of human excreta are the technologies which will permanently overcome the ascariasis problem. It seems unlikely that agents for the biological control of helminth transmission stages, including the eggs of *A. lumbricoides*, in the soil (Lysek, 1963; Waller, 1993) will be able to cope with the problem.

7.5. Progress and Problems

7.5.1. *Emerging Drug Resistance*

Control strategies centred on anthelminthic treatment with drugs tested and developed by the research-based pharmaceutical industry are at risk of being threatened by the emergence of populations of intestinal nematodes that have become resistant to the compounds used to control them and so reduce morbidity. Theoretical studies, supported by evidence from the development of pesticide resistance, indicate that it is only a matter of time before drug resistance appears (Comins, 1984; WHO, 1996b). The expected emergence of drug resistance depends on five assumptions: (i) that helminth populations are closed so there is no gene flow; (ii) that widespread anthelminthic treatment is

applied; (iii) that treatment is homogeneous; (iv) that two alleles are involved; (v) that continual selection pressure is applied. The crucial factor is the generation time of the nematode species of interest and from five to 100 generations could be required before drug resistance would emerge.

Drug resistance is judged to be present when there is a greater frequency of individuals within a population able to tolerate curative doses of a compound than in a normal population of the same species. Furthermore, resistance is a heritable character (Prichard *et al.*, 1980; Taylor, 1992). In practical terms, detection of the heritable aspect of the problem is particularly difficult not only because the intestinal nematodes are difficult to maintain in the laboratory but also because the bioavailability of the drug may have been altered either by events in the host or by events during its manufacture.

There is now abundant circumstantial evidence from many locations to show that drug resistance has developed in the strongylate nematode parasites of domesticated animals (see Taylor, 1992; Coles *et al.*, 1994). Although there is a relatively large number of anthelminthic drugs available for use in domesticated animals, many of the drugs such as the benzimidazoles are closely related and so multi-resistant lines of parasites become selected. Strains of *Haemonchus contortus* are now known from South Africa showing resistance to benzimidazoles, ivermectin, closantel and rafoxanide (Van Wyk and Malan, 1988).

There is also direct evidence to explain the molecular and genetic mechanism of benzimidazole resistance in *H. contortus*. Roos *et al.* (1990) studied DNA polymorphisms in the geonome of larval and adult *H. contortus* known to be either susceptible or resistant to benzimidazole compounds. Benzimidazole anthelminthic drugs act by binding to the nematodes' tubulin thereby impairing protein secretion, glucose uptake and microtubule formation (Taylor, 1992). Roos *et al.* used this knowledge to prepare tubulin DNA probes and concluded that benzimidazole-resistant *H. contortus* possessed an altered or reduced complement of β-tubulin genes which would mean that resistant worms would have a reduced capacity for binding the benzimidazole anthelminthic drug and then being affected by it when offered to the host at normal therapeutic levels.

Is there any evidence to indicate that anthelminthic-resistant *A. lumbricoides* or other species of intestinal nematode are now infecting humans? From the results of a study carried out in Mali, De Clercq *et al.* (1997) implied that there was some evidence to suggest that mebendazole-resistant *Necator americanus* is now present in the Sikasso region of that country. This study involved 103 infected participants who were subdivided into various groups (treated, placebo, control) and in whom the course of the infection was followed by counting eggs in stool samples. The authors state that single-dose (500 mg) mebendazole gave disappointing results but they may be premature in assuming that drug resistance is present. The study design and sample sizes

must take account of the highly overdispersed numbers of worms per host (Anderson and May, 1991). Unless treatment and control groups are large there will be every chance that the numbers of worms in each group will be significantly different leading to serious problems in the data analysis. Reynoldson *et al.* (1997) compared the efficacy of albendazole and pyrantel pamoate in a coastal aboriginal community in Western Australia. Twenty-nine individuals known to be infected with *Ancylostoma duodenale* were offered treatment with one or other of the two drugs. Seven days after treatment stool samples were examined and, while albendazole was found to have cleared hookworms completely, pyrantel pamoate was observed to have had no significant effect. Both these studies involved standard *in vitro* egg-hatch assays which further indicated variable responses to the drugs involved. Both groups of authors expressed strong opinions about the use of mebendazole and pyrantel pamoate. In the abstract of their paper wrote De Clercq *et al.* (1997) '. . . mebendazole should not be considered a drug of choice in the mass treatment of hookworm infections in this region [Sikasso] of Mali' while Reynoldson *et al.* wrote in the abstract of their paper '. . . that locally *A. duodenale* is resistant to pyrantel and . . . should not be considered the drug of choice at this dose rate [10 mg/kg body weight] in the treatment of hookworm infections (*A. duodenale*) in endemic regions'.

While these strong statements may reflect the widespread anxiety that exists over the threat of drug resistance, there is also the need for further research in the laboratory and community before it is agreed that anthelminthic drug resistance exists in the intestinal nematodes which infect humans. High priority should be given to develop protocols for the detection of genuine drug resistance as opposed to perturbations in drug efficacy. Perhaps an even more difficult task will be to provide front-line health workers with guidelines to help them report suspicions of emerging drug resistance; important first steps will be the introduction of training and resources for regular monitoring of drug quality and drug efficacy in community-based control programmes. For those workers particularly concerned about ascariasis, it is encouraging to note that at the time when his review was accepted for publication, Taylor (1992) had found no reports of drug-resistant *A. suum* in pigs.

7.5.2. *Measures to Offset Drug Resistance*

If it be accepted that current systems for using anthelminthic drugs will select for and promote intestinal nematode populations which are resistant to these drugs then measures must be developed to delay and offset this eventuality. The fundamental objective of a control programme based on chemotherapy is to achieve a sustainable, significant reduction in morbidity due to ascariasis

and other forms of soil-transmitted helminthiasis. This objective does not require eradication of the worms. One of the advantages of targeting treatment at high risk groups in the community (school-age children in the case of ascariasis; see Section 7.2) is that other infected individuals remain untreated and the genes of their worms will serve to dilute those of worms exposed to anthelminthic drugs because they had infected members of the target group. Results from a study in Bangladesh have indicated that populations of *A. lumbricoides* tend to be quite restricted to particular communities (Ibrahim *et al.*, 1994). By analysing enzyme polymorphisms in 117 adult *A. lumbricoides* obtained by expulsion chemotherapy from eight children living in different households in an urban slum, Ibrahim *et al.* found the worms to be of similar genetic composition and to come from a randomly mating population.

A considerable body of experience in coping with drug resistant nematodes has been gained by veterinarians (see Taylor, 1992). Reducing the frequency of anthelminthic treatment by dosing at intervals greater than the nematode's generation time helps to preserve drug-susceptible alleles. In the livestock industry, a balance has to be struck between the extent of this interval, which ensures effective action against the parasite and an economically acceptable loss of productivity. Such an approach raises important ethical considerations when humans are involved. If there are as few as four recommended anthelminthic drugs for use against *A. lumbricoides,* hookworms and *T. trichiura* (Section 7.2), how much morbidity can knowingly be tolerated in order to extend the functional usefulness of the drugs?

Changing the drug of choice during a control programme should also serve to delay the selection of drug-resistant parasites. Veterinarians have already found that the annual rotation of levamisole and ivermectin for the treatment of sheep in the UK is a means of dealing with benzimidazole-resistant nematodes (Taylor *et al.*, 1990). Using drugs in combination is another option to be explored for delaying the onset of drug resistance. McKenna (1990) concluded that the use of a benzimidazole–levamisole combination could delay the expected emergence of drug resistance.

The development of new anthelminthic drugs for human use would be a major advance in the effort to control morbidity due to ascariasis. Some drugs new for human use might be found in the eight benzimidazole compounds currently used by veterinarians in addition to albendazole and mebendazole (Taylor, 1992). Before any such drug could be recommended for human use at the community level there would need to be a thorough evaluation of its efficacy in relation to the results of toxicity tests, reports of side effects and so on (WHO, 1996a). Entirely new drugs might be synthesized such as nitazoxanide, a nitrothiazole benzamide which is already known to be effective against a variety of bacteria, protozoa and helminths including *Toxocara canis* infecting dogs (Rossignol and Cavier, 1976; Murphy and Friedmann, 1985; Rossignol *et*

al., 1998). Development of nitazoxanide or other drugs for use in impoverished developing countries may be curtailed by commercial decisions. Perhaps medicinal plants will hold the key to sustaining ascariasis control programmes.

7.5.3. *Traditional Medicine*

For very many people living in developing countries, where resources to support modern medical care are extremely limited (World Bank, 1993), traditional medicine remains an important option for any form of sustainable health care.

(a) *Traditional Chinese medicine (TCM).* Around half of the world's cases of infection with *A. lumbricoides* are located in China (Peng *et al.*, 1998a) which has a flourishing and well accepted system of traditional medicine. Medicinal plants, especially in China, form a valuable resource for the successful treatment of many ailments including infections of *A. lumbricoides* and other species of soil-transmitted nematode (Ou Ming, 1989). Some 7000 species of medicinal plants are used for various purposes in China. These plants have been used for centuries and must be known to be effective otherwise people would not rely or them or accept them. The prescriptions must have been found to be safe and have minimal, if any, side effects. They are locally accessible to communities in remote rural areas; there is no need for complex delivery and storage systems (WHO, 1989). Also, there is a wealth of experience and knowledge in the system of TCM about how to use these plant prescriptions (Ou Ming, 1989; Zhang and Wu, 1991) and reports of resistance to individual prescriptions have not yet appeared. Currently, the World Health Organization is preparing a series of monographs giving pharmacopoeial summaries of quality assurance, chemical assays, active chemical constituents, purity requirements, clinical applications, pharmacology, contraindications, precautions and posology.

One example can serve to show the value of TCM in the treatment of ascariasis. Biliary ascariasis (Section 2.2.3; Table 4) is one of the common causes of biliary disease in China (Cheng, 1993) with 12% of such cases being ascribed to the presence of *A. lumbricoides* in the common bile duct (Zhao, 1984). The application of TCM has now led to a highly successful and conservative form of treatment with oral doses of pills prepared from a decoction of *Fructus Mume* (Ou Ming, 1989). In a recent review, Zhou *et al.* (1999) report on 4167 cases of biliary ascariasis which were treated by prescriptions from TCM. Of these, 4060 were fully cured without surgery and most within a period of 2.5 days in hospital.

(b) *Medicinal plants in Africa.* African communities have long established traditions for using plants in the treatment of nematode infections. For example, Barnish and Samai (1992) recorded 10 prescriptions made from indigenous

plants in use for the treatment of roundworms by the Mende people of Sierra Leone. Torlesse (1999) found that the Temne people in the same country have not abandoned their confidence in plant remedies for the treatment of helminth infections and the relief of anaemia during pregnancy. Kightlinger *et al.* (1996) observed in a forest community in rural Madagascar that treatment with traditional anthelminthic prescriptions made from local plants (*Chenopodium ambrosioides*) resulted in statistically significant falls in faecal egg counts of *A. lumbricoides*. Most importantly, Kightlinger *et al.* noted that the people considered roundworms to be loathsome and harmful and that the use of medicinal plants was an indication of a 'felt need' to tackle the problem. Harnessing this 'felt need', with the cooperation and leadership of traditional birth attendants, traditional pharmacists and herbalists and others of established influence in the community, may offer the best chance for sustainable control actions. In sub-Saharan Africa, where ascariasis continues to thrive, better use of traditional medicines may offer the only affordable approach to controlling worm-induced morbidity given the desperate shortage of funds for health care.

8. ECONOMIC ASPECTS OF ASCARIASIS CONTROL

If economics is the study of scarce resources (Evans, 1992), economists must surely find the distribution of financial support for public health problems in developing countries to be a challenging and fruitful field for investigation. Basically there are two approaches to the allocation of resources for health care in this context. First, decisions may be based on the results of cost–benefit analyses and secondly, decisions may be based on conclusions reached from cost-effective analyses. Inevitably there is some overlap between the two approaches and both are dependent on numerous assumptions because data required by the analyses are either lacking or inadequate. Some of these gaps for ascariasis and other soil-transmitted helminthiases have been overcome by predictions from the model framework developed by Anderson, May and their colleagues.

8.1. General Considerations

In theory, a cost–benefit analysis would seek to demonstrate in financial terms the net gain to a community resulting from a given expenditure on a health intervention programme. Public health planners would then argue that priorities should be determined such that maximum benefit is obtained from the expenditure. For example, a cost–benefit approach could be attractive in the case of hookworm disease where relatively small expenditure to relieve iron

deficiency anaemia in adult men might be followed by relatively large and measurable gains in the product from some labour-intensive activity (Crompton and Stephenson, 1990). The problems and difficulties inherent in attempting to assess the benefits of spending resources on disease control have been set out by Dunlop (1984); the particular impediment to accuracy is the quality of available information. However, the exercise is useful for the managers of control programmes because they are provided with a checklist of points of importance.

An early attempt to devise a model whereby the health impact of different disease problems might be assessed quantitatively was produced after research in Ghana (GHAPT, 1981). The results were expressed in terms of 'days of healthy life lost' and, not surprisingly, malaria and measles featured at the top of the list because premature death is the most important determinant for most diseases when considering days of healthy life lost. Using the GHAPT system, ascariasis, which is essentially a debilitating and chronic rather than killer disease, does not feature in a list of 48 health problems. Intestinal obstruction, a problem which involved *A. lumbricoides*, was ranked at 37 in the list and was estimated to cost Ghana 4950 days of healthy life lost per 1000 persons per year compared with 32 567 for malaria (GHAPT, 1981).

The World Bank (1993) proposed that cost-effectiveness in health care could be quantified in terms of disability-adjusted life years (DALYs). This unit, which is calculated as the present value of the future years of disability-free life that are lost as the result of premature deaths or cases of disability occurring in a particular year, has been used extensively by Murray and Lopez (1996, 1997). So in sub-Saharan Africa, with a population of 510 millions in 1990, 293 million DALYs would have been lost through ill-health with 10.8 million being due to malaria and 1.8 million to all types of worm infection. Calculation of DALYs must have been a most intriguing exercise for the team involved and the summarized results (see Box 1.3, World Bank Report, 1993) provide an invaluable summary of the global and regional burden of disease. The use of DALYs has been criticized because of (a) their relation to ethical aspects of health care, (b) the inadequacy of data, (c) the multifactorial nature of morbidity and mortality and (d) the need to validate the methodology (Murray and Lopez, 1997; Sayers and Fliedner, 1997). It is difficult to imagine application of the DALY method at the local level where a health problem occurs and needs to be evaluated in the competition for meagre resources.

8.2. Practical Applications

An early attempt to estimate the financial costs of endemic ascariasis in Kenya and the savings to be made following expenditure on a national control programme was made by Latham *et al.* (1977) and Stephenson (1984). These

authors were well aware of the shortcomings of their approach and the need to regard their conclusion with great caution. However, assuming that 25% of the Kenyan population was infected with *A. lumbricoides* in 1976, they calculated that the infection was costing Kenya US $5.15 million annually and that the anthelminthic drugs for periodic deworming would have cost US $0.8 million at that time. Their cost–benefit ratio of 1 : 6.4 does not take account of drug delivery or the fact that a vertical problem aimed at ascariasis in isolation is not a viable proposition in sub-Saharan Africa. Conversely, the ratio could become more persuasive of intervention because the treated population would have gained some relief from hookworm disease and trichuriasis if an effective broad spectrum anthelminthic drug were used.

In the case of ascariasis and some other helminth infections, all available information indicates that health intervention should be directed at children who experience most morbidity and who can be reached through primary schools. Warren *et al.* (1993) and Evans and Guyatt (1995) have set out schemes for comparing the cost-effectiveness of delivering anthelminthic chemotherapy to communities afflicted with soil-transmitted helminthiases. The key objective is to find out how to achieve the greatest reduction in morbidity for the minimum cost of intervention. Major decisions to be taken include whether diagnostic screening should be undertaken and if so whether intensity should be estimated and whether treatment should be offered to everybody or be targeted at a group in the community. These options and combinations of them are discussed in quantitative detail by Warren *et al.* (1993).

Warren *et al.* then simulated a cost-effectiveness scheme targeted at children and designed to reduce morbidity as a result of treatment with praziquantel (for schistosomiasis) and albendazole (for soil-transmitted helminthiases). Features of their proposal are given in Table 15. Taking the maximum cost per child per year as US $1.8 (Table 15), the proposed intervention appears to offer extremely good value especially when the gains in DALYs resulting from such a programme amount to as much as US $33 per DALY gained. However, current figures show that US $12 is available per capita annually for all aspects of health care in a typical developing country (UNICEF, 1998). It is doubtful whether a developing country could afford to spend US $1.8 out of 12 on school-based deworming.

Holland *et al.* (1996b) published results of a cost-effective analysis based on three anthelminthic treatment regimens designed to reduce morbidity due to ascariasis. The analysis applied to three rural villages in Nigeria where one village had been offered mass (universal) treatment, one treatment targeted at schoolchildren and one treatment given only to selected individuals known to harbour high worm burdens (Asaolu *et al.*, 1991). The interventions were aimed at reducing morbidity and it was therefore assumed that a significant reduction in *Ascaris* egg count (epg stools) would be accompanied by a

Table 15 Economic aspects of a 10-year school-based programme designed simultaneously to reduce morbidity caused by schistosomiasis and soil-transmitted helminthiases in children (Data abstracted from Warren *et al.*, 1993).[a]

	Praziquantel	Albendazole
Doses per 1000 children	4 000	8 000
Cost per 1000 children[b]	2 000	1 000
Minimum delivery cost per 1000 children	5 000	
Maximum delivery cost per 1000 children	15 000	
Minimum cost per child for 10 years	8	
Maximum cost per child for 10 years	18	

[a] Details of the assumptions about prevalence, intensity, compliance and treatment intervals at the start of the intervention are given by Warren *et al.* (1993).
[b] All costs are given in US$.

Table 16 Measures of cost-effectiveness for different forms of anthelminthic intervention against ascariasis in rural Nigerian communities (Holland *et al.*, 1996b)

	Treatment strategy[a,b]		
	Village 1-SELECTIVE (high intensity only)	Village 2-TARGETED (children)	Village 3-MASS (all comers)
Total cost[c,d]	12 490	3 956	4 701
Number treated with levamisole	36	194	455
Cost per reduction of 1000 epg	5 004	611	451
Cost per person treated	347	20.4	10.3

[a] Defined by Anderson (1989); now modified by WHO (1996b).
[b] See Asaolu *et al.* (1991).
[c] All costs are given in Naira.
[d] Includes transport, technical labour for sampling, screening and treatment, supplies and drug costs.

reduction in morbidity. Measures of cost-effectiveness are set out in Table 16. Despite its limitations, the results show mass treatment to be the most cost-effective procedure. The treatment targeted at the schoolchildren is apparently twice as costly but two important points must be made. First, the *Ascaris* egg counts fell in untreated individuals in village 2, presumably because their exposure to infective eggs had declined (Asaolu *et al.*, 1991). Secondly, there is a potentially long-term benefit for the community in treating the children because they are at an age which is crucial for their physical and intellectual

development (Warren *et al.*, 1993). More operational research is needed to provide health managers in developing countries with simple and robust methods for measuring and comparing the cost-effectiveness of different interventions.

9. ORIGINS OF THE HUMAN–*ASCARIS* ASSOCIATION

How and when the host–parasite relationship between humans and *Ascaris* began are questions which ought to excite the curiosity of helminthologists. Porter (1997) in his treatise entitled *The Greatest Benefit to Mankind*, which traces the medical history of humanity from antiquity to the present, writes 'Many of the worst human diseases were created by proximity to animals . . . Settlement helped disease to settle in . . . Parasitologists and palaeopathologists have shown how the parasitic roundworm *Ascaris*, a nematode growing over a foot long, evolved in humans, probably from pig ascarids . . .'. Porter does not cite the evidence for his view but Kliks (1983) wrote 'Available evidence suggests that the most common contemporary soil-transmitted helminths of man, *Ascaris* and *Trichuris*, are human-adapted strains originating in pigs concurrent with the development of sedentary village life in Neolithic times'. Peng *et al.* (1995, 1998b) and Anderson and Jaenike (1997) have brought together various strands of circumstantial and molecular evidence to encourage the speculation that the human–*Ascaris* relationship might have emerged as humans began the process of domesticating wild boars (*Sus scrofa*). Anderson and Jaenike (1997) sought to identify whether humans or pigs were the ancestral hosts of *Ascaris* by applying molecular techniques to examine nuclear diversity in worms recovered from humans in Bangladesh, Madagascar, Peru and Guatemala and from pigs in the Philippines, Switzerland, Scotland, Peru and Guatemala. Their findings are not clear and better resolution might be obtained by further sampling but they concluded that the marginally greater genetic diversity in the nuclear genome of worms from pigs suggests that pigs served as the ancestral hosts. Peng *et al.* suggested that China was a possible site for this event, although there are no reasons for assuming that the domestication of pigs was confined to China.

Hominids are known to have existed for perhaps half a million years in China; on present evidence *Homo erectus* (Peking man) was established there many thousands of years ago and current theories indicate that *H. sapiens* emerged from the *H. erectus* stock (Leakey, 1994). *Homo erectus* lived as a hunter and it is interesting to note that at a site occupied by *H. erectus* at Choukoutien in China the bones of wild boar are among those found (Chang, 1968). Is this evidence of early contact between the ancestors of the hosts of *A. lumbricoides* and *A. suum*?

Despite numerous reorganizations during the history of her people (Spence, 1990), China has experienced a sustained civilization based on agriculture for several thousand years, as witnessed by the Neolithic villages of the Wei River and Yangtze rivers. The machinery of the Chinese state steadily evolved to cope with settled farmers rather than nomadic pastoralists who are inherently difficult to locate, count and tax (Bray, 1984; An Zhimin, 1989). Although there is great diversity within China, there is a single written language, equally understandable to every literate person and a diet in which rice and pork predominate (Reader, 1988).

The domestication of animals began with the wolf about 12 000 years BP and that of the wild boar (*Sus scrofa*) probably began a few thousand years later in Western Asia and in other places after that (Clutton-Brock, 1987). There is general agreement that today's domesticated pig (*Sus domesticus*) has been selected from wild boar (*Sus scrofa*) and the process probably began as it had done with the taming of wolves, since wolves and boars would be attracted to human settlements for scavenging on domestic waste (Lever, 1985; Bokonyi, 1989). According to studies reviewed by Davis (1987), all domestic pigs examined to date possess 38 chromosomes but wild boar from Western Europe have 36 while those in Asia and Far East have 38. Assuming that the modern distribution of boar karyotypes was similar in antiquity, the modern domestic pig probably originated somewhere between the Balkans and the Far East. Davis (1987) states that it is likely that the pig was independently domesticated in China but as yet we have little evidence from that part of the world. There is, however, some archaeological evidence to suggest that in China the pig may be as old a domesticated animal as the dog (An Zhimin, 1989). At an early Neolithic site in northern China, with a radiocarbon date of around 8000 years BP, remains of *Sus domesticus* (domestic pig) and *Canis familaris* (domestic dog) have been found together (Chow, 1989).

Humans understandably have close affinities with chimpanzees and gorillas (Diamond, 1992; Wood, 1996) and so we might expect that these three types of primate would share several infections. However, qualitative information suggests that humans harbour surprisingly few of the helminth infections found in other primate species (Coombs and Crompton, 1991). After an investigation of helminth infections in non-human primates, Orihel (1970) proposed that an infection such as *A. lumbricoides* in a non-human primate host should be referred to as an anthroponosis or disease of humans transmitted to animals. Various authors have also concluded that common human helminth infections found in non-human primates have often been acquired through contact with infected humans. In an appraisal of the social history of humans and disease, Karlen (1995) concluded that during recent human evolution we have provided new ecological niches for microbes by cultivating fields and domesticating animals. Karlen cited observations to indicate that humans now share about 300 infections

with species of domesticated animal and stressed that our helminth infections have been acquired from livestock and pets. Apparently, we share 42 diseases with domesticated pigs (Karlen, 1995). All this interaction between infectivity and susceptibility perhaps began to occur in recent times following events when humans largely abandoned the life of being hunter-gatherers and became settled in farming communities (Peng *et al.*, 1998b). Importantly, for the argument that modern humans have acquired *A. lumbricoides* as a consequence of the domestication of the wild boar, infections of what we currently know as *A. suum* are regularly found in wild boar and feral pigs (Barutzki and Richter, 1990; Schauss *et al.*, 1990; Barutzki *et al.*, 1991; Eslami and Farsad-Hamdi, 1992).

The proposal that the present day human–*Ascaris* relationship may have originated in China could be investigated further by making a comparative survey of the current number of infections found in pigs and humans in the main centres of human population. We might expect strong correlations between the range and frequency of infections shared between humans and pigs in China; that would not be in any way conclusive but it would help to support the view that human–*Ascaris* associations could have started several thousand years ago in China. Recently, Wood (1996) warned that the more we know about human evolutionary history, the more complex it becomes. Exactly the same cautious approach is needed for the consideration of the evolutionary history of human–parasite relationships. The invasion of *Homo sapiens* by *Ascaris* may well have taken place on several occasions in various locations but the extensive prevailing infections between humans, *Ascaris* and pigs in China certainly offer a tantalizing prospect about the origins of *Ascaris* infections (Peng *et al.*, 1998b).

10. CONCLUSION

Sufficient evidence now exists to demonstrate that ascariasis is a significant public health problem causing debilitating morbidity which impairs the growth and development of several hundred million children in developing countries. Ascariasis merits a major control effort not on its own but as part of a drive against the major soil-transmitted nematodes. A modest effort is already in progress, often with financial support from non-governmental organizations and other major donors. The challenge now is to devise protocols and systems to help public health planners in developing countries use their meagre resources to implement and sustain the progress made in the control of morbidity due to soil-transmitted nematode infections. Modern chemotherapy, beset by problems of costs, quality, counterfeiting and even drug resistance, may not offer a long-term approach to helminth control. In many communities the sustainable strategy may emerge from a blend of traditional medicine and modern knowledge.

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Index

Page numbers in *italic* indicate figures and tables.

Contents of Volumes in This Series

Volume 38

Volume 39

Volume 40

Part I *Cryptosporidium parvum* and related genera

Part 2 *Enterocytozoon bieneusi* and Other Microsporidia

Part *3 Cyclospora cayetanensis* and related species

Volume 41

Volume 42

Volume 43

Volume 44

Volume 45

Volume 46

Volume 47

Volume 48