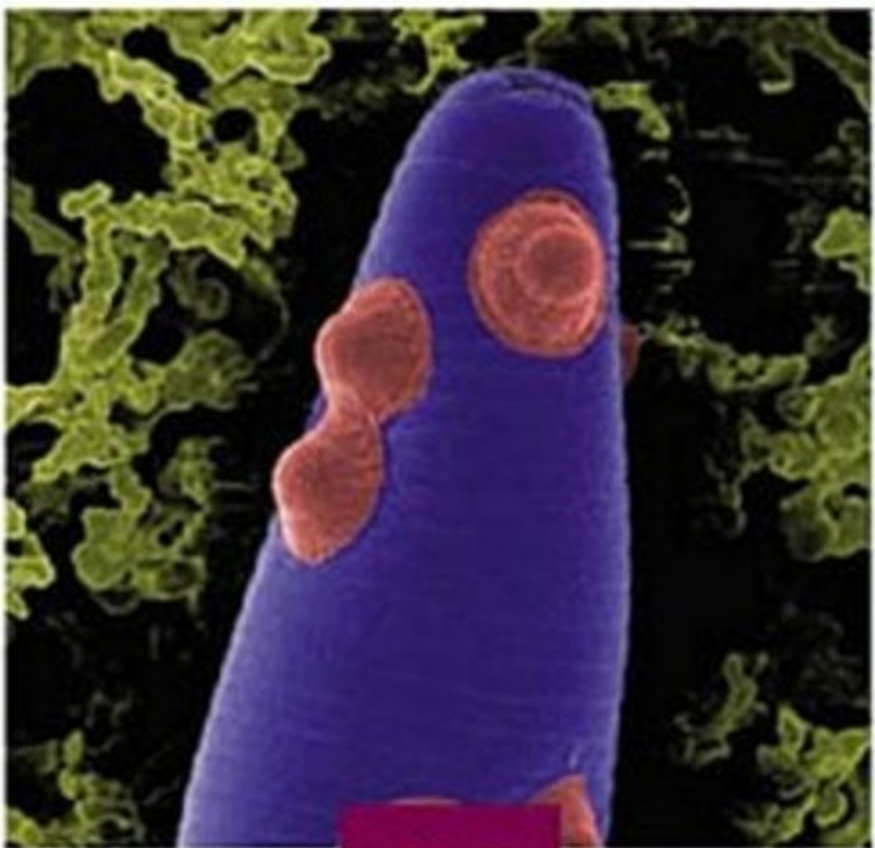
ADVANCES IN PARASITOLOGY

Natural History of Host-Parasite Interactions



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PREFACE

This thematic volume of *Advances in Parasitology* was conceived as a result of a symposium held at the Linnaean Society in the autumn of 2007, for which I had the pleasure to convene together with Dr David Rollinson, to mark the tercentenary of Linnaeus's birth in combination with the Centenary celebrations of both Imperial College and the Royal Society of Tropical Medicine and Hygiene. The symposium was extremely successful and highly attended, and we were delighted that almost every speaker was willing to subsequently contribute their papers to this volume.

Dobzhansky wrote in his famous essay, ''Nothing in biology makes sense except in the light of evolution'', yet this truth so often appears to be ignored within the medical and biomedical disciplines. Evolution and co-evolution are the foundations of biology, and biology is the foundation of medicine and public health. For example, co-evolution has epidemiological implications, particularly in the context of emerging and re-emerging diseases. If co-evolution imposes constraints on susceptibility and pathogenicity, those constraints may no longer hold when new host-parasite associations emerge or ancient associations are disrupted, affecting both the magnitude and severity of disease outbreaks. There may also be indirect effects of changes in the range of parasites to which a host population is exposed through altering the selection pressures on existing parasites. Likewise, altering host genetics, especially by selective breeding for resistance to a particular parasite, could also affect selection pressures on other parasites. Understanding how parasites respond to evolved changes in host characteristics may also provide a good model for their all too apparent potential to respond to other kinds of change, such as the use of new drugs or vaccines to combat disease. Evolutionary theory has, therefore, an important role to play in both the interpretation of host and parasite dynamics and the design and application of disease control programmes.

This volume brings together a range of articles from scientists from different fields of research and/or disease control, but with a common interest in studying the biology of a variety of parasitic (in its broadest sense) diseases. In so doing, we aim to present what evolutionary thinking can contribute to an integrated understanding of the processes shaping host-parasite interactions and control.

The volume starts with a selection of papers on viruses, which, although not always classified as parasites in the strictest sense, can in fact be termed the 'ultimate parasite'. The paper by Rebecca Payne and colleagues illustrates how the human immunodeficiency virus (HIV)

epidemic, of current key concern in terms of global health, also provides a rare opportunity to examine in detail the initial stages of a host-pathogen co-evolutionary struggle in humans. In particular, the role of human leukocyte antigen (HLA) class I and the cytotoxic T-lymphocyte (CTL) response in controlling HIV replication is described (which may also help explain how some individuals are able to elicit long-term control (>25 years) of HIV replication during the course of natural infection), as is the extent to which HIV has already adapted to those HLA class I molecules and those CTL responses that are most effective in viral suppression. It becomes evident that viral mutations that enable HIV to evade the CTL response are already accumulating in populations where the selecting HLA molecules are highly prevalent, indicating the dynamic and shifting nature of the battle currently being played out between HIV and human populations. Indeed, the sequence variation observed at a population level is not random variation, but appears to be the consequence of Darwinian selection operating in the context of the host immune response. As a result, HIV may be predicted to become adapted at a population level to those immune responses currently identified as mediating control, which is clearly a major anxiety in relation to vaccine design and future control.

Host-viral interactions are also the focus of Paul Ewald's paper, in particular the role they may play in human cancers. Infectious causation of human cancer was generally considered non-existent during the first half of the 20th Century, and later reported only as noteworthy exceptions to the general rule. Indeed, by the mid-1970s, only one human cancer was widely acknowledged to be caused by uni-cellular or sub-cellular parasites, that of endemic Burkitt's lymphoma, potentially in response to co-infection of *Plasmodium falciparum* with Epstein Barr Virus (EBV). However, with, in part, the current growing recognition of the molecular mechanisms of pathogen-induced oncogenesis, this paper describes how pathogens, particularly viruses, either alone or in synergy with other infectious agents, may be major initiators of oncogenesis for many, if not most, cancers, with the traditional mutation-driven processes becoming dominant only after this initiation. Many supporting key examples are presented, such as, for instance, the mouse mammary tumour virus (MMTV), a causative agent of some human breast cancers. Because cancers are so devastating and their treatment is often both gruelling and marginally effective, a solid record of preventing infection-induced cancers demands that the full spectrum of infectious causation of cancers be characterised and accurately understood. Now about 15–20% of all human cancer are accepted by the World Health Organization as being caused by parasitism and it is likely that such cases will become more and more apparent.

The role of parasites in a range of chronic conditions is followed in the paper by Thierry Lefèvre and colleagues, when considering the diversity

and evolution of manipulative strategies in host-parasite interactions. In this case the altered behaviour of the host is a phenotype of the parasite and is controlled by the parasite's genes, and hence may be termed part of the parasite's 'extended phenotype'. In this review the authors examine the mechanisms by which parasites are known to control the behaviour of their hosts and describe novel methodologies for future research, such as the need for more molecular and specifically proteomic techniques for determining the genetic basis of manipulation. The authors propose that parasites do not necessarily manipulate the brain of their hosts in the way a puppeteer controls a puppet, delicately tweaking only those neural circuits responsible for specific behavioural traits. Instead they suggest that certain parasites appear to strike the host's brain with a number of diffuse and widespread effects, some of which induce changes in host behaviour. A range of examples are described, such as the case of the contrasting pathological phenotypes (aggressive vs. paralytic forms) displayed in human rabies between individuals, even following bites and hence viral 'strain' from the same rabid dog, thereby implying a differential role of the host immune response in enabling such virally altered host behaviour. The authors also highlight how, in the vast majority of cases, our level of understanding of such host-parasite interactions is far from complete, despite the fact that parasites are believed by some (admittedly non-parasitologists) to be simple organisms.

Hilary Hurd's paper on the evolutionary drivers of parasite-induced changes in host life-history traits continues and expands upon several of the general themes raised in the preceding papers. Key concepts under consideration here relate to whether changes in host reproductive fitness are by-products of infection, parasite manipulations, host adaptations, mafia-like strategies and/or host compensatory responses. Her paper focuses on the reproductive fitness of insect hosts and vectors, in particular that of tapeworms and beetles and malaria infections in anopheline mosquitoes. Evidence is put forward for both a manipulator molecule of parasite origin and for host-initiated regulation. This paper again highlights how it is imperative that evolutionary theories must now be supported by empirical evidence gained from studying the molecular, biochemical and physiological mechanisms underlying changes in host life-history traits, ideally using organisms that have evolved together and that are in their natural environment.

Katrin Hammerschmidt and Joachim Kurtz's paper considers host-parasite interactions in parasites with complex life cycles, and hence those that require two or more consecutive invertebrate and vertebrate hosts. Despite the fact that so many parasites, including those of profound medical and veterinary importance, have complex life cycles, our understanding of the evolution of complex life cycles is currently still in its infancy. This paper describes in detail recent research into the

immunological interaction of such a parasite, the model tapeworm *Schistocephalus solidus*, with its two intermediate hosts, a cyclopoid copepod and the three-spined stickleback. The data presented indicate that immunological interactions between host(s) and parasite(s) are relevant factors influencing not only parasite establishment and growth, but potentially also behavioural manipulation of the hosts. In complement to the Lefèvre *et al*. paper above, these authors elaborate upon the ''extended phenotype'' concept to include the proximate physiological causes, whereby parasitised hosts can truly be seen as ''deeply modified organisms''.

Turning towards more field-based evolutionary and epidemiological studies, Judith Smith examines one of our most ubiquitous parasites, *Toxoplasma gondii.* The paper describes how this parasite's, again complex, life cycle has become adapted to exploit multiple routes of transmission through a sexual cycle in the definitive host and asexually in the intermediate host. While such alternative routes may operate synergistically to enhance transmission, this paper illustrates how they might also provide a vehicle for selection, leading to partitioning of strains in the environment, including potential differences in shifts from sexual to asexual transmission between epidemiological regions.

Alison Dunn's review considers the fate of (non-human) parasites during a biological invasion and their impact on both native and invasive hosts, asking whether parasites can directly or indirectly mediate invasion success. Using illustrations from a range of studies focusing on parasitism in amphipod invasions, this paper describes how, for example, an introduced species may either lose its parasites as a result of the introduction, introduce novel parasites to hosts in the new range and/or acquire parasites from its new environment. Furthermore, this paper highlights how, as a result of local adaptation, parasites tend to have a differential effect on native versus invading hosts, which will be a key determinant for the outcome of any invasion and its impact on the recipient community.

Fiona Mathews' paper then considers the importance of a detailed understanding of the ecology of zoonotic diseases in wildlife, both in terms of predicting their success and managing their control. More than two-thirds of emerging, or re-emerging, infectious diseases are thought to originate in wildlife. Despite this, co-ordinated surveillance schemes are rare, and most efforts at disease control operate at the level of crisis management. This review examines the pathways linking zoonoses in wildlife with infection in other hosts, using examples from a range of key zoonoses including European bat lyssaviruses and bovine tuberculosis. The paper also describes how, while the vast majority of efforts to control zoonoses in wildlife hosts rely on culling strategies, the alternative, and

potentially more successful, approach is to understand the factors leading to disease outbreaks in the first place and to manage these instead.

Issues of the importance of understanding host-parasite interactions for disease control, in this case biocontrol by a bacterium *Pasteuria penetrans*, a hyperparasite of root-knot nematodes (*Meloidogyne* spp.), are also illustrated in the paper by Keith Davies. It is only relatively recently with the development of industrialised agriculture that plant parasitic nematodes have been recognised as an important constraint on crop production. For the majority of their evolutionary history, plant parasitic nematodes have been part of a multi-trophic interaction between their plant host and their natural enemies. This paper discusses the reasons why bacterium-nematode surface interactions are likely to hold the key to understanding host-specificity and evolutionary dynamics in this system, and presents some genomic insights into potential solutions for future bio-control.

In terms of direct disease control of human parasites (and hence where public health measures can be seen as a major interaction by a host on their parasites), Alan Fenwick's paper documents how recent shifts in global health policy have led towards the implementation of mass chemotherapeutic control programmes at the national scale in previously 'neglected' countries, such as those within sub-Saharan Africa. However, while celebrating the rapid success achieved to date by such programmes, in terms of reduced infection prevalence, intensity and associated human morbidity, it is acknowledged that evolutionary change in response to drug selection pressure may be predicted under certain circumstances, particularly in terms of the development of potential drug resistance. Theoretical and empirical data gained to date thereby serve to highlight the importance of careful monitoring and evaluation of parasites and their hosts whenever and wherever chemotherapy is applied and where parasite transmission remains.

The paper by María-Gloria Basáñez and colleagues then focuses on one of these neglected tropical diseases, onchocerciasis, in relation to its blackfly (*Simulium*) vector, with particular reference to the transmission dynamics, density-dependent interactions, evolutionary implications and ultimately control of human onchocerciasis. The authors examine evidence to suggest that *Onchocerca* may exploit interactions to enhance its transmission and discuss the consequences on onchocerciasis transmission of local adaptation in *Onchocerca-Simulium* complexes. Mathematical models to coalesce and interpret current data and help identify optimal control strategies are introduced. The authors describe in detail the prospect that drug resistance may potentially become a public health concern, and how future genetically structured mathematical models combining population dynamics and genetics may provide insights into evolutionarily stable strategies for different host-parasite complexes.

Jacob Koella and colleagues' paper concludes this volume with a consideration of novel aspects for control of a major disease of public health importance, specifically that of microsporidians as so-called 'evolution-proof' agents of malaria control. Despite substantial control efforts control, malaria remains one of our most serious and deadly diseases. While some of the problems in controlling malaria are socio-economic, others are biological, in particular those relating to this parasite's intense transmission and the emergence and spread of resistance of the malaria parasites and their mosquito vectors against most of the chemicals used to attack them. The authors question the potential success of proposed future malaria control agents. For example, even following the development of effective vaccines, subsequent strong novel selective pressures may be likely to induce the parasite to develop escape-mutants. Indeed, certain vaccines may even by predicted to lead to more virulent parasites. Likewise the potential effectiveness of novel genetic strategies, aimed to use either transformed sterile male mosquitoes or to drive genes for resistance to infection linked to a transposable element through populations, is also questioned. New methods for control are therefore desperately needed, although such methods would be useful only if they are effective (i.e., decrease transmission substantially) and evolutionarily sustainable (i.e., evolution-proof, in that they prevent evolution from eroding efficacy). These authors propose microsporidian parasites that infect mosquitoes as one potentially effective and sustainable agent for malaria control, and describe in detail a range of recent studies to support this. The authors conclude that, while the evolution of resistance may be inevitable, with a solid understanding of the host-parasite systems involved the failure of control need not be.

Therefore, this collection of papers covers a wide range of systems, exemplified by a broad spectrum of micro-and macroparasites, impacting humans, domestic and wild animals, and plants. It illustrates the importance of evolutionary considerations and concepts, both as thinking tools for qualitative understanding or as guiding tools for decision making in major control programmes. We thank the support of the editorial team of *Advances in Parasitology* and hope that our readers will enjoy this volume as much as we have enjoyed preparing it.

JOANNE P. WEBSTER

CHAPTER 1

HLA-Mediated Control of HIV and HIV Adaptation to HLA

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Abstract The human immunodeficiency virus (HIV) epidemic provides a rare opportunity to examine in detail the initial stages of a host-pathogen co-evolutionary struggle in humans. The genes encoding the human leukocyte antigen (HLA) class I molecules have a critical influence in the success or failure of the immune response against HIV. The particular HLA class I molecules expressed by each individual defines the type of cytotoxic T-lymphocyte (CTL) response that is made against the virus. This chapter describes the role of HLA class I and the CTL response in controlling HIV replication, and discusses the extent to which HIV has already adapted to those HLA class I molecules and CTL responses that are most effective in viral suppression. It is evident that viral mutations that enable HIV to evade the CTL response are indeed already accumulating in populations where the selecting HLA molecules are highly prevalent, indicating the dynamic and shifting nature of the evolutionary interplay between HIV and human populations.

1.1. INTRODUCTION

1.1.1. Epidemiology

Since human immunodeficiency virus (HIV) was identified in the early 1980s as the causative agent of acquired immunodeficiency (AIDS), the number of people living with HIV has relentlessly increased. In 2007, 2.5 million new infections were reported. Since 1981 there have been an estimated 25 millions deaths (UNAIDS WHO AIDS epidemic update 2007).

1.1.2. Control of HIV: Progress and challenges in therapeutics

The introduction of anti-retroviral therapy (ART) has resulted in a decrease in mortality and morbidity among HIV-infected subjects. However, access to ART is still very limited in most resource-poor countries where the epicentre of the pandemic is located. Thus, while the prospect of an effective HIV vaccine remains bleak, it has never been so needed. The failure, in 2007, of one of the most promising T-cell-based vaccine candidates, which aimed either to protect against HIV transmission or to lower viral loads in vaccinees who became infected (Sekaly, 2008; Steinbrook, 2007; Watkins *et al.*, 2008), has prompted the HIV scientific community to confront the limitations in our knowledge of what constitutes protective T-cell immunity.

There are multiple challenges to creating an effective HIV vaccine that is able to elicit, what are believed to be, the immune responses likely to contain HIV, namely, broad neutralising antibodies and strong cytotoxic T-lymphocyte (CTL) responses. Perhaps the greatest of these challenges is the sequence variability of HIV, a hallmark of the virus, which results from the high error rate of the viral reverse transcriptase (Preston *et al.*, 1988). Thus, from an HIV vaccine perspective, effective immune responses need to be induced against a vast range of different, albeit closely related, viruses. In addition, the sequence variation observed at a population level is not random variation, but is to some extent the consequence of Darwinian selection operating in the context of the host immune response. Within a particular infected individual, the virus adapts to the immune responses generated against it by selecting viral amino acid sequence changes that reduce immune recognition of HIV and these increase in frequency, ultimately replacing the wild-type virus. Following transmission of virus to a new recipient, some of these viral adaptations persist. In this way, HIV may become adapted at a population level to those immune responses currently identified as mediating control. This is clearly a major anxiety in relation to vaccine design.

1.1.3. Control of HIV: Successful immune responses

In spite of the success with which HIV can evade the host immune response, some individuals are able to elicit long-term control of HIV replication during the course of natural infection. Individuals infected more than 25 years ago have been identified, who have levels of plasma HIV so low that the virus is undetectable by even the most sensitive assays. It is valuable to understand which immune responses are responsible for controlling HIV replication in this way. This review focuses on the central role played by T-cell immunity in control of HIV infection. In particular, we address the impact of viral evasion of CTL ($CD8^+$ T cell) responses through the selection of mutations that reduce or abrogate the recognition of virus-infected cells by CTLs—so-called 'CTL escape'—on immune control of HIV in the individual, and at a population level. Finally, we discuss the implications for vaccine design of CTL as a major driving force of HIV evolution.

1.2. $CD8^+$ CYTOTOXIC T LYMPHOCYTES (CTL) AND CONTROL OF VIRAEMIA

In the first few weeks of untreated adult HIV infection, the level of viraemia typically rises to around 10^7 HIV ribonucleic acid (RNA) copies per millilitre (ml) of plasma. This subsequently declines during the following few

weeks by 10^2-10^3 copies per ml of plasma to a relatively stable viral set-point with a median of around 30,000 copies per ml. The particular set point established in each individual HIV-infected person is strongly predictive of the time it will take for that person to progress to AIDS; lower viral set-points predict slower progression to AIDS and higher viral set-points predict more rapid progression (Mellors *et al.*, 1996). For instance, a viral set point of 30,000 copies per ml of plasma is predictive of AIDS progression in approximately 10 years in the absence of ART.

There are several lines of evidence to indicate the central role of CTL in control of HIV replication. First, the temporal association between the appearance of HIV-specific CTL responses and the decrease in viral load during acute infection suggests the importance of CTL in the establishment of viral set-point (Borrow *et al.*, 1994; Koup *et al.*, 1994). This observation was confirmed by studies in the Simian Immunodeficiency Virus (SIV)-macaque model, in which depletion of circulating CTL with anti-CD8 monoclonal antibodies resulted in a loss of control of viraemia in both the acute and chronic phase (Jin *et al.*, 1999; Matano *et al.*, 1998; Schmitz *et al.*, 1999).

A second line of evidence to support the role of CTL in immune control of HIV is the association between certain HLA class I molecules and disease outcome (Carrington and O'Brien 2003; Goulder and Watkins 2008; Kiepiela *et al.*, 2004). CTL are able to recognise HIV-infected target cells because the infected cells present fragments of HIV proteins in the peptide-binding groove of cell-surface HLA class I molecules. Recognition of the HIV peptide/HLA complex on the target cell, by the T-cell receptor (TCR) of the CTL results in the release of cytokines, chemokines and molecules, such as perforin and granzymes, that affect the rapid lysis and apoptosis of the infected target cell. The HLA region, which is situated on the short arm of chromosome 6, is the most polymorphic of the entire human genome (Mungall *et al.*, 2003). This extraordinary diversity ensures that a wide range of pathogen-derived proteins can be presented for recognition by CTL. The disease outcome from HIV, and other infectious diseases that are contained by CTL, is thus critically dependent on the particular protein fragments presented by HLA class I molecules. Which HIV proteins form successful targets for CTL and which form apparently useless targets for CTL is further discussed below.

In the context of HIV, the reason that different HLA molecules can be associated with particular disease outcomes may be due to differences in the peptide-binding groove of the HLA molecules and hence the different fragments of HIV peptides that are presented for recognition by CTL. For example, HLA-B*57, which is associated with successful control of HIV infection, typically binds peptides that carry either a tryptophan or a phenylalanine (both large, hydrophobic residues) at the carboxy-terminus of the peptide. HLA-B*27, also associated with slow progression, only binds peptides that carry an arginine at position 2 (Marsh *et al.*, 2000).

A third line of evidence suggesting the importance of CTL in control of HIV infection is the demonstration that the selection of particular CTL escape mutations can precipitate loss of immune control (Barouch *et al.*, 2002; Feeney *et al.*, 2004; Goulder *et al.*, 1997). Taken together, and as discussed further below, these studies indicate the strong causal link connecting particular HLA molecules and the resulting CTL responses with effective control of HIV replication.

1.3. DISEASE OUTCOME MEDIATED BY CTL

1.3.1. Effective CTL responses and dominant role of HLA-B

The association between HIV immune control and expression of certain HLA class I molecules is most striking for alleles located in the HLA-B locus. For example, HLA-B*27, HLA-B*57 and HLA-B*51 have been associated with successful control of HIV infection whereas HLA-B alleles such as HLA-B*5802 and HLA-B*3502 have been associated with rapid disease progression (Honeyborne *et al.*, 2007; Kiepiela *et al.*, 2004; Leslie *et al.*, 2006; O'Brien *et al.*, 2001). The HLA-B locus is the most polymorphic of the three major HLA class I loci, with 817 alleles described compared with 486 HLA-A alleles and 263 HLA-C alleles (IGTM/HLA database). Indeed, the HLA-B locus is the most polymorphic region in the entire human genome reflecting the fact that this is a site of exceptionally strong balancing selection (Belich *et al.*, 1992; Watkins *et al.*, 1992) and the vital role played by HLA-B in immune protection from pathogens whose control is dependent upon CTL.

The mechanism by which particular HLA-B alleles mediate viral control of HIV provides a crucial clue to understanding which CTL responses need to be induced by an effective HIV vaccine. Recent studies have suggested that a critical factor linking these protective HLA-B alleles is the fact that they all present epitopes from within the HIV Gag protein, whereas HLA alleles associated with a lack of immune control present no, or few, Gag epitopes (Matthews *et al.*, 2008). Indeed, several population studies of HIV infection have shown that an increased breadth of Gag-specific $CD8^+$ T-cell responses correlates with decreased viral load, irrespective of HLA type, while no correlation has been observed for non-Gag-specific responses (Edwards *et al.*, 2002; Geldmacher *et al.*, 2007; Honeyborne *et al.*, 2007; Kiepiela *et al.*, 2007; Klein *et al.*, 1995; Masemola *et al.*, 2004; Novitsky *et al.*, 2003; Riviere *et al.*, 1989, 1995, Zuniga *et al.*, 2006). Studies of immune control of SIV in several different macaque models also suggest a key role for Gag as an immune target (Goulder and Watkins, 2008). In short, a broad Gag-specific CTL response

is associated with effective control of HIV infection, and lack of a broad Gag-specific CTL response with ineffective control.

1.3.2. CTL responses targeting HIV Gag

There are three main reasons why CTL targeting of Gag might be particularly important in immune control of HIV infection (Fig. 1.1A and B). Gag ('group-specific antigen') is so-called because of the conservation of the amino acid sequence of the Gag protein between members of the lentiviridae, which include HIV and SIV. Gag, in fact, is comprised of several proteins, the largest of which is the capsid protein (p24 Gag), which forms the central conal core of the virus and which contains two copies of viral RNA and the viral enzymes such as reverse transcriptase. p24 Gag is particularly conserved—that is, there is very little amino acid sequence variation in this protein from one HIV-infected individual to another, whereas for many of the other HIV proteins, in particular Envelope and the Accessory and Regulatory proteins (Tat, Rev, Nef, Vif, Vpr and Vpu), there is much inter-individual viral sequence variability. The lack of sequence variability observed in p24 Gag, despite the vast potential for variation generated by the error-prone reverse transcriptase, implies strong purifying selection driving conservation of amino acid sequence in this protein.

The implication drawn from the lack of sequence variability in p24 Gag is that amino acid sequence changes within this region are not well tolerated by the virus. Accumulating evidence supports this hypothesis, demonstrating that CTL escape mutations selected in Gag result in significant fitness costs to the virus (Brumme *et al.*, 2008). This has been demonstrated in particular in relation to the HLA-B*57- and HLA-B*5801-restricted epitope TSTLQEQIGW (Gag 240-249, TW10), the HLA-B*57-restricted epitope, KAFSPEVIPMF (Gag 161-171, KF11) and the HLA-B*27-restricted epitope, KRWIILGLNK (Gag 262-271, KK10) (Crawford *et al.*, 2007; Martinez-Picado *et al.*, 2006; Schneidewind *et al.*, 2007). Similarly, escape mutations within the SIV Gag epitopes restricted by Mamu-A*01, Mamu-90120-5 and Mane-A*10 have also been shown to reduce viral replicative capacity significantly (Fernandez *et al.*, 2005, 2007; Friedrich *et al.*, 2004; Kawada *et al.*, 2006; Kobayashi *et al.*, 2005; Matano *et al.*, 2004; Mothe *et al.*, 2003; O'Connor *et al.*, 2003; Tsukamoto *et al.*, 2008).

The second reason why Gag may be an important CTL target for effective immune control of HIV is that Gag is highly immunogenic. More CTL responses are directed against Gag than any other HIV protein (Kiepiela *et al.*, 2007). The immunogenicity of Gag is likely to relate to its abundance in virus-infected cells (Briggs *et al.*, 2004), and to the findings from studies in the SIV model that have shown early presentation of epitopes from Gag and Pol proteins on the surface of

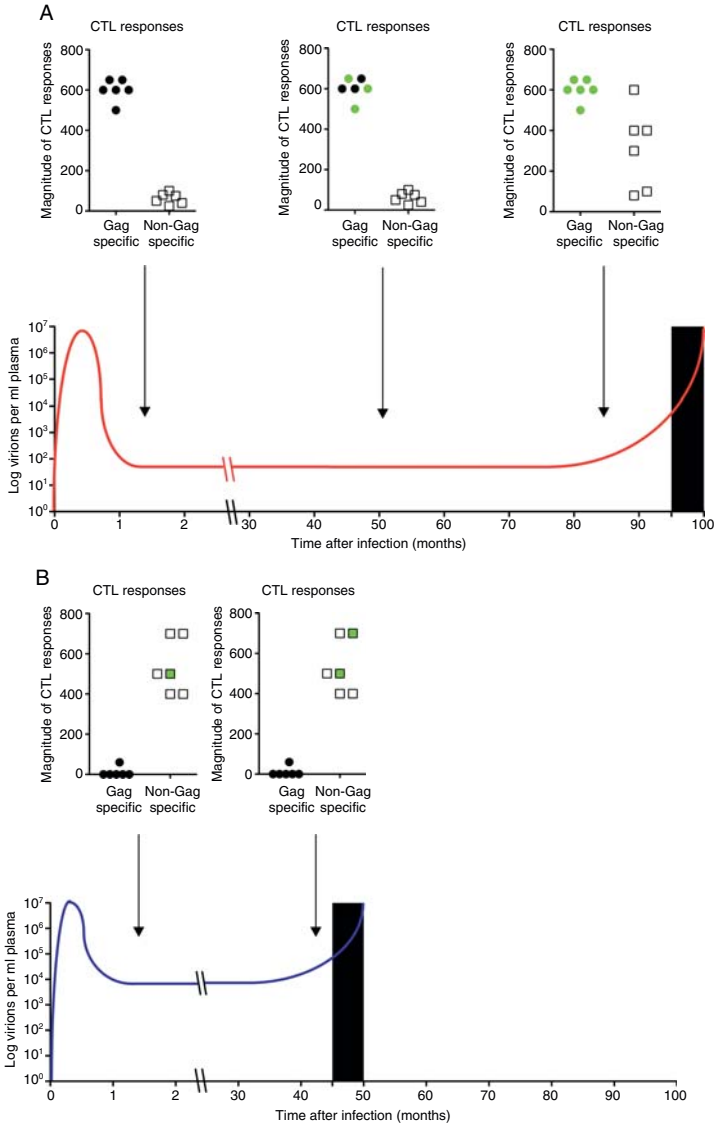


FIGURE 1.1 Schematic representation of cytotoxic T-lymphocyte (CTL) immune control in HIV infection. (A) Subject with a predominately Gag-specific CTL response. Gag-specific CTL (black circles) are temporally associated with a reduction in viral load in acute infection and low viral set point. Selection of viral escape mutations by Gag-specific CTL (grey circles) results in continued immune control due to the cost of mutations to viral fitness. Selection of multiple Gag-specific mutations, including

infected cells (Sacha *et al.*, 2007a,b). This latter observation represents the third important reason why Gag may be a critical target for effective immune CTL responses. Presentation of HIV epitopes from the Regulatory and Accessory proteins and from Envelope proteins appear to occur after *de novo* synthesis of progeny virions, some 12 h following infection of the target cell. However, Gag and Pol proteins are sufficiently abundant to be processed and presented directly from the incoming virions in as little as 2 h post-infection. The advantage of early Gag epitope presentation is that CTL recognition and targeting of infected cells may occur early enough to prevent the production and release of progeny virus and hence prevent HIV dissemination. Additionally, once Nef has been synthesised, MHC class I expression on the cell surface is down-regulated and the subsequent decreased presentation of HIV-epitopes on the cell surface reduces CTL killing (Collins *et al.*, 1998). However, although Gag and Pol are both presented early, there is nonetheless a substantially greater abundance of Gag within the infected cell (20-fold higher Gag vs Pol) (Shehu-Xhilaga *et al.*, 2001), on the cell surface presented by HLA molecules (20-fold higher, Gag vs Pol) (Tsomides *et al.*, 1994), and in HIV particles (1,000–1,500 molecules present in mature HIV particle) (Briggs *et al.*, 2004). In conclusion, protective CTL responses in HIV infection are linked to HLA-B expression and to the ability of particular HLA-B molecules to present multiple Gag epitopes. HIV protein sequence conservation, T-cell immunogenicity and early presentation are likely to be the crucial factors explaining the effectiveness of Gag-specific CTL responses.

1.4. IMMUNE ESCAPE—VIRAL ESCAPE MUTATIONS FROM CTL

1.4.1. Impact of HIV escape mutations within a host

Although the concept of CTL escape was first described in 1991 (Phillips *et al.*, 1991), the consequences of such mutations on disease progression have remained unclear. There is only one clear-cut example of an

compensatory mutations that restore viral fitness followed by the appearance of non-Gag-specific CTL (open boxes), is associated with loss of immune control and progression to AIDS (black bar). (B) Subject with a predominately non-Gag-specific CTL response. Non-Gag-specific CTL (open boxes) are temporally associated with a reduction in viral load in acute infection and high viral load set point. Selection of viral escape mutations by non-Gag-specific CTL (grey boxes) has no effect on viral load or viral fitness. Loss of immune control and progression to AIDS typically occurs earlier than in subjects who target multiple Gag-specific CTL (black bar).

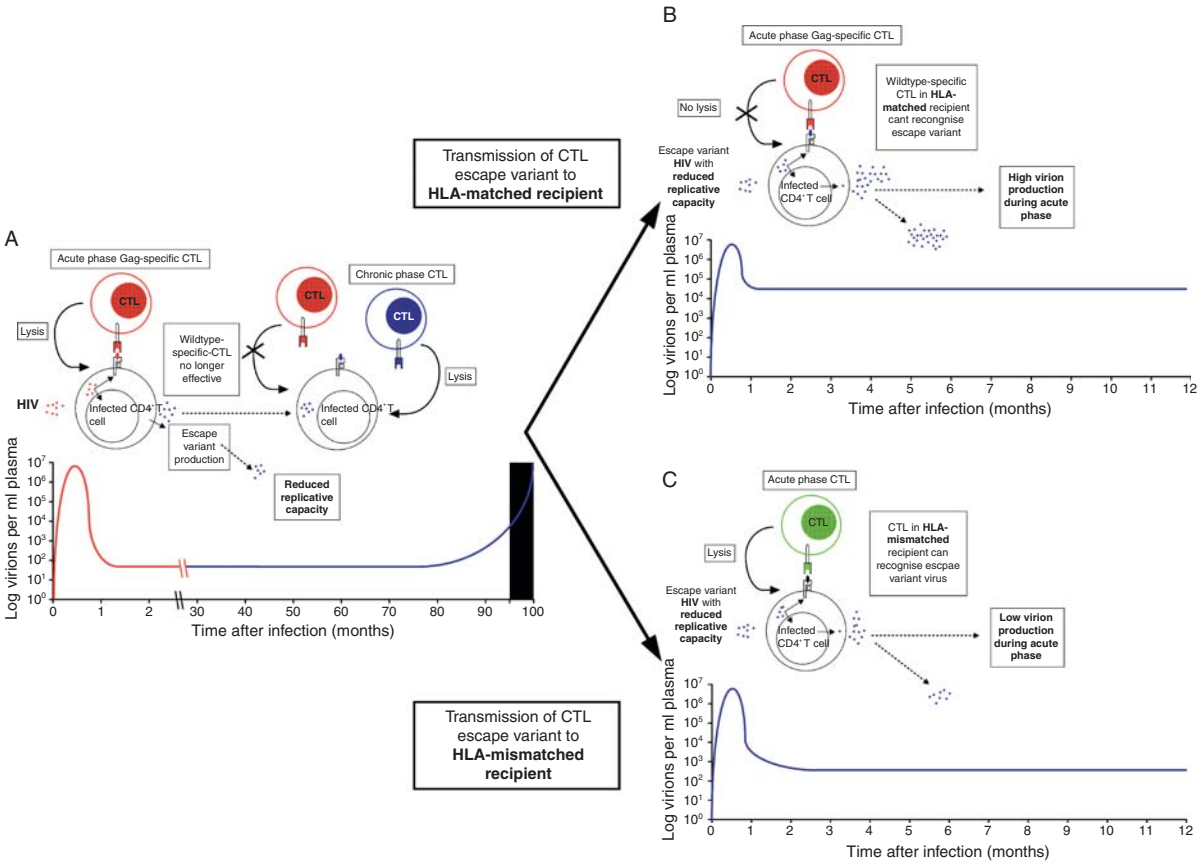
HIV-specific CTL escape mutation precipitating disease progression—the R264K escape mutation in the HLA-B*27-restricted Gag epitope KRWIILGLNK (KK10, Gag 263-272) (Feeney *et al.*, 2004; Goulder *et al.*, 2001). Indeed, the majority of CTL escape mutations do not significantly affect viraemia (Kiepiela *et al.*, 2007; Matthews *et al.*, 2008). However, a recent study of more than 700 HIV-infected South African study subjects showed that the greater the number of escape mutations in Gag associated with each HLA-B allele, the better the immune control linked to that HLA-B allele (Matthews *et al.*, 2008). These data support the notion that CTL are effective against HIV either by rapid recognition and killing of virus-infected cells, or by driving escape mutations that partially cripple the virus. For the reasons described previously (see Section 1.3.2), Gag is the HIV protein most likely to enable CTL to deliver effective hits to the virus via either of these means.

1.4.2. Impact of HIV escape mutations transmitted to a new host

The transmission of viral escape mutations that were selected in response to the donor's HLA alleles might be anticipated to have two particular consequences for the newly infected recipient. For recipients HLA-matched with the donor, the transmission of viruses encoding escape mutations in epitopes commonly presented by the matched HLA allele might be disadvantageous, since those epitopes would be unavailable to the recipient. For recipients HLA-mismatched with the donor, the transmission of viruses encoding escape variants does not affect epitope presentation by the recipient, but might be advantageous due to reduce viral replicative capacity. (Fig. 1.2).

1.4.2.1. Horizontal transmission

In an analysis of 114 adult transmission pairs in Zambia, there is evidence of both of these effects operating. The viral load of the newly infected recipients was negatively correlated with the number of HLA-B-associated, Gag-specific escape mutations transmitted (Goepfert *et al.*, 2008). This association was strongest when the recipients were infected with virus that did not carry mutations in epitopes presented by their own HLA alleles. Thus, Gag-specific mutations are not only of clinical benefit to the individual donor but also to an HLA-mismatched recipient upon transmission, in all likelihood due to the fitness cost of the transmitted escape mutations. A complementary study in South Africa showed that, despite reversion of transmitted Gag-specific escape variants in HLA-mismatched recipients, a significantly lower viral load and higher CD4 count was observed at 12 months post-infection in these recipients, than compared to HLA-mismatched recipients who became infected with the


A
Acute phase Gag-specific CTL
CTL
Lysis
Wildtype-specific-CTL no longer effective
Chronic phase CTL
CTL
CTL
Lysis
HIV
Infected CD4+ T cell
Infected CD4+ T cell
Escape variant production
Reduced replicative capacity
Log virions per ml plasma
Time after infection (months)
Transmission of CTL escape variant to HLA-matched recipient
B
Acute phase Gag-specific CTL
CTL
No lysis
Wildtype-specific CTL in HLA-matched recipient cant recognise escape variant
Escape variant HIV with reduced replicative capacity
Infected CD4+ T cell
High virion production during acute phase
Log virions per ml plasma
Time after infection (months)
Transmission of CTL escape variant to HLA-mismatched recipient
C
Acute phase CTL
CTL
Lysis
CTL in HLA-mismatched recipient can recognise escpae variant virus
Escape variant HIV with reduced replicative capacity
Infected CD4+ T cell
Low virion production during acute phase
Log virions per ml plasma
Time after infection (months)

wild-type virus (Chopera *et al.*, 2008). Since there is a link between viral load during primary infection and viral load set-point (Kelley *et al.*, 2007), it is likely that escape mutations that incur a fitness cost to the virus will be of long-term benefit to an HLA-mismatched recipient, despite subsequent reversion, by reducing viraemia during acute infection. While these studies have highlighted the potential clinical benefit of transmission of viruses with HLA-selected escape mutations, it is important to emphasise that only Gag-specific escape mutations mediated this effect. Indeed transmission of HLA class I-associated Nef escape mutations had no impact on the viral load of recipients (Goepfert *et al.*, 2008).

1.4.2.2. Vertical transmission

Mother-to-child transmission (MTCT) represents a special case where donor and recipient share at least half of their HLA alleles. MTCT involves infection of children who are HLA matched with their transmitter mother through at least one of the two HLA-B class I molecules expressed in the mother; in some cases, both HLA-B alleles are shared, especially where the HLA-B alleles concerned are highly prevalent in the population. Indeed, it is possible that one factor contributing to the more rapid progression to HIV disease observed in infected children compared to adults is the likelihood that the transmitted virus, adapted to the mother's HLA alleles, is also pre-adapted to at least some of the child's HLA alleles. A small study of HLA-B*27-positive infants supports this hypothesis. This showed that HLA-B*27-positive infants, whose mothers

FIGURE 1.2 Schematic representation of cytotoxic T-lymphocyte (CTL) immune control upon transmission of Gag-specific CTL escape variants to human leukocyte antigen (HLA)-matched and HLA-mismatched recipients. (A) Subject A expresses an HLA allele, which confers a protective phenotype in human immunodeficiency virus (HIV) infection. Gag-specific CTL (red CTL) help elicit a reduction in viraemia during acute infection and establish a low viral set-point through early presentation and recognition of infected cells. Selection of Gag-specific escape mutations (blue dots) leads to a loss of recognition by the Gag-specific CTL but results in a fitness cost to the virus. CTL responses arise during the chronic phase of infection (blue CTL). (B) Transmission of virus with Gag-specific escape mutations (blue dots) to an HLA-matched recipient that shares the protective HLA allele. Gag-specific CTL in HLA-matched recipient (red CTL) are unable to recognise viral variant resulting in a higher virion production during acute infection and consequently a higher viral set-point than expected for subjects expressing the protective HLA allele. (C) Transmission of virus with Gag-specific escape mutations to an HLA-mismatched recipient with no protective HLA alleles. Acute-phase CTL in HLA-mismatched recipient (green CTL) can recognise viral variant (blue dots). Combined effect of CTL activity and reduced replicative capacity of transmitted virus results in a lower viral load set-point than expected for subjects expressing non-protective HLA alleles.

also expressed HLA-B*27 and who had transmitted the escape mutation in the critical KK10 Gag epitope, progressed relatively rapidly. In contrast, an HLA-B*27-positive child whose mother did not express HLA-B*27, and who therefore transmitted a virus encoding the unmutated KK10 epitope, was able to target CTL to the KK10 epitope and thereby attain successful immune control (Feeney *et al.*, 2004; Goulder *et al.*, 2001). However, the sharing of HLA alleles between donor and recipient may only be relevant if key HLA-B alleles associated with control of HIV are involved.

1.5. HIV EVOLUTION AND IMMUNE SELECTION

1.5.1. Origin and evolution of HIV

HIV-1 emerged in humans after transmission of non-pathogenic SIV from chimpanzees (*Pan troglodytes*) in central Africa, with three separate transmission events suggested by the phylogenetic division of HIV-1 into groups M, N and O interspersed between SIV lineages (Wain *et al.*, 2007). The origins of M-group viruses—the most prevalent, and the most diverse, of the three groups—can be traced to a common ancestral sequence calculated to have arisen in the early 1930s (Korber *et al.*, 2000).

The continued genetic diversification of HIV-1 is attributable to the combined influence of selectively neutral genetic drift (Shriner *et al.*, 2004), and positive selection pressure imposed on the virus—for example, by host immune responses (Leslie *et al.*, 2004, 2005; McMichael and Klenerman, 2002; Moore *et al.*, 2002, Wain *et al.*, 2007) or by ART (Lemey *et al.*, 2005; Little *et al.*, 2008). Here we focus on two dominant, and interrelated, evolutionary forces with a strong impact on HIV phylogeny; descent from a common ancestral sequence—termed 'founder effect' (Bhattacharya *et al.*, 2007)—and immunological pressure imposed by HLA-selection (Matthews *et al.*, 2008; Moore *et al.*, 2002; O'Brien *et al.*, 2001; Rousseau *et al.*, 2008).

M-group viruses are phylogenetically sub-divided into clades, genetic sub-groups that are defined by sequence differences at the nucleotide level. There are several strands of evidence to suggest that these clades arose from founder strains in Africa rather than diverging subsequently as a consequence of immunological selection pressure in different human populations (Peeters and Sharp 2000; Vidal *et al.*, 2000). First, the starburst appearance of the M-group phylogenetic tree suggests near simultaneous evolution of viral sub-types from SIV transmission events (Rambaut *et al.*, 2001). Second, analysis of envelope sequences from HIV-1 strains circulating in central Africa reflects the enormous genetic diversity of HIV in this region, encompassing sequences from all M-group

clades (Nkengasong *et al.*, 1994; Rambaut *et al.*, 2001; Vidal *et al.*, 2000). Additionally, study of phylogeny and geographic routes of dissemination of the different clades suggests direct transmission events from African founder viruses (Gilbert *et al.*, 2007; McCutchan *et al.*, 1996; Thomson *et al.*, 2007; Vidal *et al.*, 2000). The presence of distinct viral sub-clades in different geographical locations—for example, the characteristic clustering of C-clade Indian viruses—is also suggestive of descent from a single common ancestor (Gaschen *et al.*, 2002). Likewise, viruses circulating in Thailand form a distinct sub-cluster within E-clade, as well as bearing similarities to strains that have been identified in central Africa (McCutchan *et al.*, 1996).

1.5.2. HLA footprints and HIV evolution

Distribution of HLA class I alleles is geographically diverse, with different human populations having marked differences in phenotypic frequency of HLA alleles (Goulder and Watkins, 2008). Phylogenetic clustering of HIV taxa in different geographical areas may therefore also relate to the common selection of particular 'HLA footprints' that arise in response to prevalent alleles in the human population. Perhaps the best-studied example is the strong selection pressure imposed by HLA-B*57 (Leslie *et al.*, 2004; Martinez-Picado *et al.*, 2006). HLA-B*5703 is the most common sub-type of HLA-B*57 in sub-Saharan Africa, and this allele is associated both with the most effective control of HIV and also with the greatest number of escape mutations in Gag (Crawford *et al.*, 2007; Matthews *et al.*, 2008; Fig. 1.3). As a consequence of this shared external selection pressure, even genetically disparate viruses exposed to the same HLA selection pressure may acquire enough shared polymorphisms to result in some degree of phylogenetic clustering (Matthews *et al.*, personal communication). In this way, HLA selects for sequence similarities, potentially driving convergent evolution in populations where the selecting alleles are common. Sites in Gag, Pol and Nef at which there are differences in amino acid consensus sequence between clades in fact

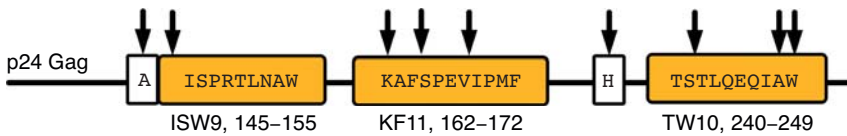


FIGURE 1.3 Schematic View of p24 Gag. Positions of HLA-B*57-restricted epitopes ISW9, KF11 and TW10 are shown in grey boxes. Mutation sites selected by HLA-B*5703 are shown by black arrows. White boxes denote positions of a processing mutation (A146X) and a compensatory mutation (H219Q).

correspond closely to those sites of HLA-mediated selection pressure (Matthews *et al.*, personal communication), suggesting that although HIV-1 clades arose as a consequence of founder effect, HLA-selection may be a determinant of on-going viral evolution.

A key question is whether the impact of this HLA-driven evolution of HIV results in an adaptation of HIV to the key HLA alleles, such as B*57, B*27 and B*51, currently central to immune control of HIV. A study of viral sequences and HLA types of more than 2,500 HIV-infected individuals from eight diverse human populations has examined whether the frequency of escape mutations in critical epitopes is accumulating in populations where the prevalence of the restricting HLA allele is high (Goulder *et al.*, in press). Overall, the prevalence of polymorphisms in well-defined epitopes correlates strongly with the phenotypic frequency of the selecting allele in the study population. For example, the prevalence of escape mutations at position 8 in the HLA-B*51 restricted epitope TI8 (TAFTIPSI, RT 128-135) is proportional to the phenotypic frequency of HLA-B*51 in the population. In Japan, where HLA-B*51 is highly prevalent, the TI8 escape mutation is present in two-thirds of the population. Similarly, Gag mutations associated with HLA-B*57 (Fig. 1.3), and associated with HLA-B*27 are strongly correlated with the prevalence of these alleles in the different populations studied. These studies suggest that, over time, the accumulation of escape mutations in these epitopes may lead to the establishment of a new consensus, replacing original population wild-type. As described previously in this review (see Section 1.4), it seems likely that the well-established associations between certain HLA alleles and control of HIV are shifting. While the consequences of these changes at this early stage of the HIV epidemic are uncertain, it is clear that the particular HIV-specific CTL responses that are currently effective in immune control of HIV may not be effective in the future. The antigen targets for the best of the immune responses may be constantly changing in sequence, but in turn, this creates new opportunities for the immune system, both for previously sub-dominant responses to become dominant, and for new responses to be induced.

1.6. SUMMARY

Successful immune control of HIV is the exception, but is well-described and achievable. Induction of these effective immune responses is the aim of a CTL-based HIV vaccine. HLA class I molecules and CTL play a central role in suppression of HIV, in particular certain HLA-B molecules such as HLA-B*57 and HLA-B*27. CTL responses targeting the HIV Gag protein are consistently associated with low viral set-point, and therefore with slow progression to AIDS. Gag-specific CTL may recognise

HIV-infected cells early in the viral life cycle and therefore typically kill virus-infected cells well before new virions are released. Mutations arising in non-Gag virus proteins that result in loss of CTL recognition of HIV-infected cells are rapidly selected and typically do not affect viral load or viral replicative capacity. Mutations arising in Gag may allow escape, but usually also incur a cost to viral replicative capacity, especially if arising in the highly conserved capsid protein p24 Gag. Accumulation of escape mutations in populations where the selecting HLA molecules are highly prevalent indicates the dynamic and shifting nature of the co-evolutionary struggle between HIV and human populations.

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CHAPTER 2

An Evolutionary Perspective on Parasitism as a Cause of Cancer

Paul W. Ewald

Contents

Abstract

For the past half-century, the dominant paradigm of oncogenesis has been mutational changes that disregulate cellular control of proliferation. Parasitic causes of cancer were first incorporated into this paradigm by suggesting mechanisms through which parasitism might increase mutational damage, such as generation of mutagenic compounds during immunological activity. The growing recognition of the molecular mechanisms of pathogen-induced oncogenesis and the difficulty of generating oncogenic mutations without first

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having large populations of dysregulated cells, however, suggests that pathogens, particularly viruses, are major initiators of oncogenesis for many if not most cancers, and that the traditional mutation-driven process becomes the dominant process after this initiation. Molecular phylogenies of individual cancers should facilitate testing of this idea and the identification of causal pathogens.

2.1. THE CANCER PROBLEM

Cancer accounts for about one-quarter of human death in wealthy countries and about one-eighth worldwide (World Health Organization, 2008). Since the discovery of the structure of DNA over half a century ago, the dominant paradigm for oncogenesis of human cancer focused on changes of human genetic material: mutations in genes that regulate cellular proliferation and migration progressively transform normal cells into metastatic cancer cells. This paradigm persists pervasively in studies that do not consider evolutionary processes as well and in evolutionary studies that view oncogenesis as an evolutionary process within individuals (Crespi and Summers, 2005; Frank, 2007; Greaves, 2008; Komarova and Wodarz, 2008; Merlo *et al.*, 2006). Consideration of the role of parasitism in oncogenesis fundamentally alters this view of cancer particularly for the earliest stages of oncogenesis.

2.2. HISTORY OF PARASITISM AND ONCOGENESIS

Although a role for parasites (broadly defined here to include the multi-cellular, cellular, sub-cellular replicating agents) was demonstrated in animal models before the demonstration of mutation-driven oncogenesis, parasites have generally been considered to be of minor importance for human cancers. One reason for marginalising parasite-induction of human cancers is that the early experimental evidence was on laboratory animals, which generated cancers rapidly after infection. Peyton Rous's demonstration of the transmissibility of cancer over a century ago studied a cancer of chickens that developed within a few weeks after inoculation with the transmissible agent, now known as Rous's sarcoma virus. No pathogen of humans seemed able to develop cancer so rapidly after infection. Infectious causation of human cancer was generally considered non-existent during the first half of the 20th Century, then a rare oddity and, most recently, noteworthy exceptions to the general rule.

For decades, infectious causes of cancers have been used in experimental studies to make cells more vulnerable to non-infectious causes

through genetic exposure to mutagens and direct molecular manipulation of genes. Infectious causation has also been credited with providing heuristic insights into the main (non-infectious) processes of oncogenesis. This marginalisation was particularly true during the first 25 years after the discovery of the structure of DNA. Over the past 30 years, however, a clearer sense of the oncogenic importance of parasitism has gradually been emerging.

2.2.1. Recognition of helminths

Trematodes were the first category of parasites to be accepted as causes of human cancer. For half a century opisthorchid trematodes, which are acquired from eating raw fish, have been linked to cholangiocarcinoma, a liver cancer originating from the bile duct cells (Hou, 1956; Manson-Bahr and Apted, 1982), and schistosome trematodes have been recognised as a cause of bladder cancer (Gelfand *et al*., 1967; Mostafa *et al*., 1999; Mustacchi and Shimkin, 1958). In the mid-1970s, however, only one human cancer was widely acknowledged to be caused by uni-cellular or sub-cellular parasites: Burkitt's lymphoma.

2.2.2. Viruses as co-factors

Endemic Burkitt's lymphoma occurs in response to co-infection of *Plasmodium falciparum* and Epstein Barr Virus (EBV). For most of the years since this association was proposed, the mechanism by which these two pathogens contribute to oncogenesis was a mystery. Recent evidence indicates that *P. falciparum* exacerbates the oncogenic effects of EBV by activating EBV replication (Chêne *et al*., 2007).

The mechanism by which trematodes contribute to cancer is still unclear. Most speculation has focused on the elevation of reactive and potentially mutagenic compounds that occurs during inflammation (Holzinger *et al*., 1999). Increases in cholangiocarcinoma prevalence have been reported during the past four decades in countries such as the United States and United Kingdom (Patel, 2001; West *et al*., 2006), where dietary exposure to opisthorchid trematodes is virtually non-existent and hepatocellular cancer has also increased. This spatial and temporal pattern implicates chemical mutagens or pathogens. Over the past decade studies have implicated hepatitis B and C viruses as risk factors for intra-hepatic cholangiocarcinoma in western and eastern countries, though the relative importance seems to vary from region to region (Donato *et al*., 2001; Lee *et al*., 2008; Shaib *et al*., 2007; Zhou *et al*., 2008). HIV is a risk factor for cholangiocarcinoma (Shaib *et al*., 2005) and for cancers with known and suspected infectious causes. These findings lend credence to the possibility that trematodes may have oncogenic

effects in synergy with viral infection, as *P. falciparum* contributes to Burkitt's lymphoma in synergy with EBV.

A similar argument applies to bladder cancer. Co-factors emphasised in the literature on bladder cancer have been largely restricted to mutagenic chemicals such as those generated by inflammation and nitrosamines (Mostafa *et al*., 1999), but oncogenic serotypes of human papilloma virus (HPV) have been associated with a bladder cancer (Kim and Kim, 1995; Moonen *et al*., 2007). This viral association raises the possibility that *Schistosoma* may interact with viruses to generate bladder cancer, though the viruses tested seem to account for at most only a small proportion of bladder cancer (Kim and Kim, 1995; Moonen *et al*., 2007).

2.2.3. The past three decades

Since the mid-1970s, acceptance of infectious causation of cancer has steadily increased through the recognition of several infectious causes of particular cancers (Table 2.1). Burkitt's lymphoma was accepted as resulting from infection soon after a causal association was proposed (Kafuko and Burkitt, 1970). The resistance to acceptance of this hypothesis was slight, probably because Burkitt's lymphoma was a rare tropical cancer that was of little interest to cancer specialists in wealthy countries. Moreover, because it requires two different pathogens, infectious causation of Burkitt's lymphoma could be dismissed as a novelty rather than a paradigm for infectious causation of cancer. The total percentage of human cancer accepted as being caused by pathogens (i.e., that attributed to Burkitt's lymphoma) during the mid-1970s was therefore less than 0.01%. Opisthorchid and schistosome trematodes were the only other parasites that were generally accepted as causes of cancers at this time. Here again their role could be dismissed as largely irrelevant to cancers in wealthy western countries, because trematode infections were rare in these countries. Worldwide the total amount of cancer accepted as caused by parasites of any kind (Burkitt's lymphoma plus trematode-induced cancers) was about 1%. Over the past three decades, this percentage has increased steadily (Table 2.1). Now about 20% of all human cancer is accepted by the World Health Organization as being caused by parasitism.

Although overviews of cancer often presume that the remaining 80% are caused by something other than parasitism, this conclusion is not justified by the evidence. A causal role for parasitism can be excluded for less than 5% of all cancer. This claim may seem dubious, given that non-parasitic causes are clearly critically important in causing some common cancers, for example, ultraviolet (UV) radiation is an important cause of skin cancer and tobacco smoke is an important cause of lung cancer (Greaves, 2000; Karagas *et al*., 2007; Kleinsmith, 2006).

TABLE 2.1 Acceptance of parasitic causes of human cancers. Years of acceptance are approximate because the transition to acceptance generally has been gradual and controversial

Cancer	Parasite	~ Year accepted	Strongly affected regions or populations
Cholangioma liver cancers	Opisthorchid trematodes	1965	East and Southeast Asia
Bladder cancer	Schistosome trematodes	1970	Middle East, South Asia, Africa
Burkitt's lymphoma	Epstein Barr virus* jointly with *Plasmodium falciparum*	1975	Equatorial Africa
Adult T-cell leukaemia	Human T-lymphotropic virus I*	1980	Africa, Japan, Caribbean, South America
Cervical cancer	HPV*	1985	Worldwide
Nasopharyngeal cancer	EBV*	1990	China, Inuit
Liver cancer	Hepatitis B* and C* viruses	1995	Worldwide
Kaposi's sarcoma	Human herpesvirus 8*	1995	Africa, Mediterranean, MSM
Stomach cancer	*Helicobacter pylori***	2000	Worldwide
Oropharyngeal cancer	HPV*	2005	Worldwide

Notes: *Pathogens that are transmitted at least in part by sexual contact or kissing. ** Mode of transmission is uncertain, but may involve sexual contact or kissing. EBV, Epstein Barr virus; HPV, human papilloma virus; MSM, men who have sex with men.

However, because all three categories of disease causation may act in concert, identification of a cause in one category cannot be used as evidence that any other category is invalid. The documented importance of inherited predispositions, environmental mutagens, and the genes they mutate therefore does not imply that infectious agents play no causal role even in these two cancers. In the case of skin cancer, low amounts of protective skin pigment is an obvious example of an inherited genetic vulnerability (Greaves, 2000; Kleinsmith, 2006) but as discussed below, evidence also implicates non-genital serotypes of HPV. In the case of lung cancer, tobacco smoke is associated with a dramatic elevation in lung infection and may systemically compromise immunological defences against infection. Unless studies search out and evaluate such infectious correlates, a causal role for infection cannot be excluded.

2.3. MECHANISMS OF ONCOGENESIS

The increasing recognition of parasitic causes of cancers and the molecular studies of their oncogenic mechanisms represent a protracted, ongoing revolution in the understanding of oncogenesis. The standard explanation since the discovery of DNA can be labelled the mutational disregulation paradigm. When the first examples of infectious causation of cancer began surfacing, they were accommodated by fitting them into this paradigm. Parasitism is often associated with a rise in reactive chemical compounds that are involved in defensive processes such as inflammation. Parasitism was therefore assumed to promote cancer because such compounds increased mutation rates. Elevation of mutation rates by immunological activity may have an important contribution to oncogenesis. However, clarification of the molecular mechanisms of viral oncogenesis indicates that viruses play a more direct role because they have evolved to compromise barriers to oncogenesis.

Cells have four critical barriers to cancer: *cell cycle arrest* keeps the cell from dividing, *apoptosis* (cell suicide) can destroy proliferating cells before they progress to metastatic cancer, *restriction of telomerase* can block oncogenesis by placing an upper limit on the total number of divisions that a cell lineage undergoes and *cell adhesion* prevents metastasis.

Viruses that are known to cause human cancer are also known to compromise each of these barriers. The viruses have evolved different mechanisms for interfering with these barriers, but all barriers are compromised by each of the best-studied viruses: human T-lymphotropic virus, hepatitis B virus, EBV, HPV, and human herpes virus 8 (Azam and Koulaouzidis, 2008; Banerjee *et al*., 2007; Boya *et al*., 2004; Brechot, 2004; Ding *et al*., 2007; Dyson *et al*., 2008; Fonsato *et al*., 2008; Gewin *et al*., 2004; Guasparri *et al*., 2008; Hayes *et al*., 2004; Hino *et al*., 2008; Knight

et al., 2005; Liu *et al*., 2005; Matteucci *et al*., 2004; Mileo *et al*., 2006; Moore and Chang, 2003; Murakami *et al*., 2005; Portis and Longnecker, 2004; Sieburg *et al*., 2004; Sinha-Datta *et al*., 2004; Subramanian and Robertson, 2002; Tungteakkhun and Duerksen-Hughes, 2008; Wang *et al*., 2004).

These viruses have apparently evolved intricate interference mechanisms because compromising these barriers fosters persistence and spread of the viruses within the human body. By causing the cells that they infect to divide, the genetic material of a virus can replicate in concert, while incurring little exposure to the immune system. By interfering with apoptosis, the virus can keep the cell from destroying the virus via cell suicide. By increasing telomerase activity, the virus can push the infected cell towards immortality, perpetuating this profitable exploitation of the host cell for resources and protection. By altering cell-to-cell adhesion, infected cells can spread to other parts of the body to facilitate further viral proliferation and transmission. This argument does not imply that pathogens benefit from lethal cancer. Rather, the breakdown of the barriers to cancer favour persistence within the host but nudge infected cells towards cancer.

Only a small proportion of the people who are infected with any one of these viruses will develop cancer. This fact indicates that any one of these oncogenic viruses is by itself insufficient to cause human cancer. Other causes must therefore contribute to oncogenesis. The common occurrence in virally induced cancers of mutations that influence proliferation and adhesion emphasises that mutations play an important role in virus-driven oncogenesis. In fact, virus-driven oncogenesis leaves the standard paradigm largely in tact for most of the process of oncogenesis, because the compromising of barriers to cancer is generally expected to be the initiating step of oncogenesis (right side of Fig. 2.1).

This expectation follows from consideration of the improbability of generating mutational dysregulation of all three barriers to proliferation

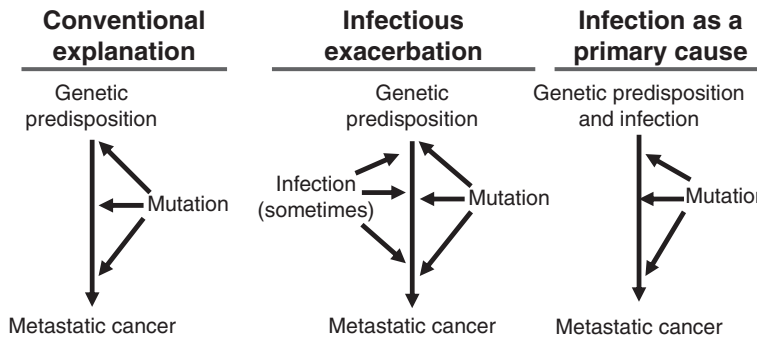

FIGURE 2.1 Alternative hypotheses for carcinogenesis.

without incurring mutations that make the cell lineage dysfunctional. Specifically, when only one barrier to cancer is compromised by mutation, opportunities for oncogenesis are extremely limited. Activation of cell replication without inhibition of cellular senescence, for example, would generate only a limited number of cellular divisions unless the cell type constituitively expressed telomerase. Similarly, inhibition of cellular senescence would be of limited value without inhibition of apoptosis, which would act to destroy the infected cell. Without infectious causation, an unlikely sequence of specific mutations would generally have to compromise these barriers without destroying the cell's viability. In contrast, oncogenic viruses generally compromise all of the barriers to metastatic cancer simultaneously. This simultaneous dysregulation allows infected cells to proliferate greatly. Once large numbers of pre-cancerous infected cells are generated, the standard arguments about cancer evolution would apply: additional oncogenic mutations that further inhibit these or other barriers to cancer would favour evolution of sub-sets of the pre-cancerous cells towards cancer. Even if the vast majority of these mutations collectively cause large numbers of pre-cancerous cells to become non-functional, oncogenesis can proceed because many other actively dividing infected cells remain to generate the rare oncogenic mutations that confer a competitive advantage through additional dysregulation of proliferation and adhesion.

This argument challenges the mutational dysregulation paradigm because it assigns a primary causal role to infection in many and perhaps most cancers. This argument predicts that cancers will occur without infection only for those cells that have the cellular barriers to cancer suppressed or shut down (e.g., stem cell or other cell types for which long series of cell divisions are necessary) or when a single mutation can compromise multiple barriers simultaneously. The retinoblastoma protein, for example, regulates cell cycle arrest, but can also facilitate apoptosis and delay cellular senescence (Sage *et al.*, 2003; Wagner and Roemer, 2005). Mutations in the retinoblastoma gene may therefore simultaneously compromise three critical barriers to cancer. This unusual tendency may explain why infection may not be necessary for the oncogenesis of retinoblastoma (Fig. 2.2). For infection-induced cancers, infectious dysregulation and mutational dysregulation are co-primary causes because oncogenesis requires both processes. In terms of practical public health benefits infectious dysregulation may be more important when infection can be prevented (e.g., through vaccination) but the causes of mutation cannot.

The tendency of oncogenic viruses to compromise so regularly the four barriers seems astonishing, especially considering that the oncogenic viruses are evolutionarily diverse and generally use entirely different viral proteins to compromise the barriers. Clearly, natural selection

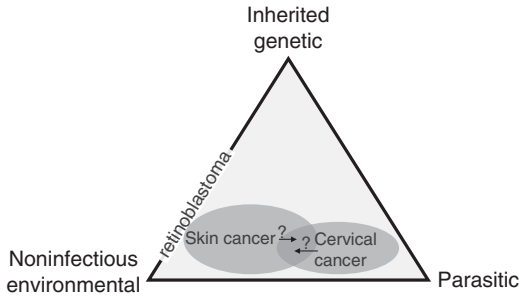

FIGURE 2.2 The triad of disease causation. The diagram emphasises joint influences of the three categories of disease causation. The placement of a disease corresponds to the relative importance of the three categories with the closest apex indicating the primary cause as defined in the text. The vertices that are more distant represent exacerbating (i.e., secondary) causes or co-primary causes of secondary importance (see text for further explanation).

must be exerting strong selection pressures on the viruses to sabotage these defences. Whatever the reason, the tendency for sabotaging the complete suite of defences seems to be more selectively advantageous for the viruses than sabotaging just one or two. The most reasonable explanation of this tendency is that the sabotaging of these barriers permits persistence and spread throughout the body, and ultimately increased transmission to new hosts.

2.4. TRANSMISSION MODES AND THE EVOLUTION OF PERSISTENCE

These arguments pertaining to transmission and persistence draw attention to the need to understand why some pathogens cause persistent infections in humans. For persistence to benefit a pathogen evolutionarily, persistent infections must still be transmissible to new hosts. However, evolution of characteristics that allow for persistent, transmissible infections in the face of the sophisticated mammalian immune systems must be difficult. This formidable evolutionary hurdle could be overcome if there is a sufficiently long evolutionary history with humans to allow for pathogens to strike upon the particular vulnerabilities of the immune system that allow persistence, or if the selective advantage of persistence is sufficiently strong to favor the evolution of mechanisms of persistence in a short evolutionary period. The selective pressure favouring persistence would be especially strong when opportunities for transmission to new hosts are infrequent. Transmission by sexual contact through genital contact or intimate kissing should generally accord with this condition.

A respiratory tract pathogen could be transmitted through coughs and sneezes to many people each day. Similarly daily transmission to many susceptible individuals could occur through diarrhoeal contamination of objects or water supplies or mosquitoes when mosquito density is high. However, opportunities for transmission sexual contact are limited by the number of new sexual partners that an individual has per day. For most people this number is very low. Sexually transmitted pathogens therefore particularly benefit from persistent infections, which allow them to remain transmissible over time periods that span multiple partner changes. A similar argument applies to pathogens transmitted by intimate kissing.

In accordance with these arguments, pathogens that have been accepted as aetiological agents of cancers are usually transmitted by genital contact or intimate kissing (Table 2.1), whereas only about 20% of all pathogens that are maintained in humans by these categories of transmission. This tendency suggests that those pathogens that will eventually be accepted causes of cancer will also tend to be transmitted during sexual intercourse or kissing. This expectation is consistent with the transmission modes of pathogens suspected of causing human cancers (Table 2.2). This association suggests where we might look for candidate pathogens of cancer, namely among those transmitted by intimate kissing or genital contact. The association also suggests how we might avoid getting cancer even for those cancers whose aetiological agents are not yet accepted or perhaps not yet even suspected.

2.5. INTERACTIONS OF CAUSES

The recognition of the importance of infectious causation and mutation in oncogenesis draws attention to the need to consider the full range of hypotheses of disease causation in any comprehensive perspective on oncogenesis. A triangle of disease causation facilitates unbiased consideration of the three general categories of causation and the interactions among them (Fig. 2.2). The placement of a disease within the triangle reflects the importance of the three categories as primary (initiating) or secondary (exacerbating) causes. Diseases are placed closest to the vertex that corresponds to the primary cause of the disease. A primary cause is defined as one that is necessary for the disease to occur. Prevention of the primary cause(s) of a disease prevents the disease. Prevention of a secondary cause will reduce the frequency or severity of disease but will not prevent the disease itself. Placement within the triangle (instead of at one vertex itself or on top of one axis) signifies that all three categories of causation contribute to oncogenesis. If two categories of causation are necessary, then the causes are considered co-primary causes and, all else being equal, the disease would be placed equidistant between the two vertices.

TABLE 2.2 Cancers that have been associated with particular parasites for which a causal role has not yet been generally accepted

Cancer	Candidate parasites	References
Colorectal cancer	*Schistosoma japonicum*	Mostafa *et al.*, 1999
Liver cancer	*Schistosoma japonicum*	Mostafa *et al.*, 1999
Merkel cell cancer	Merkel cell polyomavirus***	Feng *et al.*, 2008
Mesothelioma	Simian virus 40 (SV40)***	Cristaudo *et al.*, 2005; Yang *et al.*, 2008
Breast cancer	MMTV**, EBV*, HPV*	Bonnet *et al.*, 1999; Damin *et al.*, 2004; de Villiers *et al.*, 2005; Fina *et al.*, 2001; Kan *et al.*, 2005; Kleer *et al.*, 2002; Lawson *et al.*, 2001; Wang *et al.*, 1995
Acute lymphoblastic leukaemia	EBV*	Lehtinen *et al.*, 2003
Hodgkin's lymphoma	EBV*	Kapatai and Murray, 2007; Kutok and Wang, 2006
Non-Hodgkin's lymphomas	EBV*, SV40***	Alexander *et al.*, 2007; Kutok and Wang, 2006
Skin cancers	HPV	Andersson *et al.*, 2008; Karagas *et al.*, 2006
Oesophageal cancer	HPV*	Acevedo-Nuño *et al.*, 2004
Colon cancer	JC virus***	Hori *et al.*, 2005; Lin *et al.*, 2008
Ovarian cancer	Unknown retrovirus***, EBV*	Littman *et al.*, 2003
Prostrate	Xenotropic murine retrovirus***, BK virus***	Balis *et al.*, 2007; Fan, 2007

Notes: The references pertain to analyses of the causal role of the pathogens; * pathogens that are transmitted at least in part by sexual contact or kissing; ** mode of transmission is uncertain, but transmission in mouse host may involve genital and intermittent salivary contact; *** mode of transmission is unknown. EBV, Epstein Barr virus; HPV, human papilloma virus; MMTV, mouse mammary tumour virus.

Retinoblastoma provides an illustration. Retinoblastoma is generally considered to be caused by mutations in the retinoblastoma gene, Rb. The normal retinoblastoma protein regulates proliferation by enforcing or releasing the cell from cell cycle arrest. Mutations in both homologous copies of Rb are released from cell cycle arrest. Retinoblastoma that is associated with generation of mutations of both homologues de novo in an individual is about seven times more common than retinoblastoma associated with inheritance of one mutated Rb allele and mutation of the other homologue. If equal co-primary roles are assigned for environmentally induced mutations and inherited mutations in the latter case, and environmentally induced mutations are responsible for both mutations in the former case, retinoblastoma would be positioned on the genetic non-infectious environmental axis, closer to the environmental vertex as noted in Fig. 2.2. Alternatively, familial retinoblastoma could be positioned midway between the genetic and environmental vertices, and non-familial retinoblastoma could be positioned on the environmental vertex. If infections play some role then any of these placements would be shifted towards the infectious vertex in accordance with the role of infection.

Skin cancer is an example for which existing evidence leads to a different, more ambiguous placement. It is generally agreed that UV rays contribute to skin cancer (Greaves, 2000; Karagas *et al.*, 2007; Kleinsmith, 2006). Low amounts of protective skin pigment represent an inherited genetic vulnerability for skin cancer (Greaves, 2000; Kleinsmith, 2006). This genetic vulnerability is an exacerbating cause rather than a primary cause because dark skin colour alone will not completely protect against skin cancer. Although skin cancers are not generally considered to be caused by infection, evidence implicating non-genital serotypes of HPV as a cause of squamous cell skin cancer has accumulated over the past decade, particularly for people with suppressed immunity and the rare hereditary skin disease epidermodysplasia verruciformis (Andersson *et al.*, 2008; Jenson *et al.*, 2001; Karagas *et al.*, 2006). These considerations suggest that UV light is a primary cause of squamous cell skin cancer, hence the placement of skin cancer close to the environmental vertex. The role of genetic predispositions (genetic bases for amounts of melanin in the skin), requires that skin cancer be positioned partway towards the genetic vertex. The evidence that implicates HPV suggests that skin cancer should also be positioned partway towards the parasitic vertex. If infection proved to be an essential co-primary cause, skin cancer would need to be moved approximately half-way towards the parasitic vertex (signified by the arrow with the question mark in Fig. 2.2). Considering certain types of skin cancer separately would alter the placement; if, for example, attention is restricted to the inherited epidermodysplasia verruciformis, the evidence implicating HPV suggests a central location within the triangle.

Cervical cancer offers a third illustration. The current consensus implicates oncogenic serotypes of HPV in virtually all cervical cancers. The inability of oncogeneic HPV to cause cervical cancer in most infected women implicates a lack of exposure to environmental mutagens, genetic susceptibilities or both. Associations with cigarette smoking suggest that environmental factors are important. These considerations lead to the placement of cervical cancer partway partly towards the environmental vertex. The question mark in Fig. 2.2 suggests that proper placement may be mid-way between the parasitic and environmental vertices, if environmental mutagens are always necessary co-primary causes of cervical cancer. Allelic variation in genes associated with host defences (such as MHC genes) suggest that inherited vulnerabilities to parasitism will almost always exist, and therefore that proper placement of parasitic diseases will always involve at least some displacement from the parasitic/environmental axis. Accordingly, cervical cancer is positioned in Fig. 2.2 partway towards the genetic vertex even though the genetic bases of this vulnerability have not yet been determined.

2.6. BREAST CANCER

Consideration of breast cancer illustrates the importance of applying this integrated evolutionary perspective to cancers of uncertain causes. The most widely accepted evolutionary explanation for breast cancer focuses on the oncogenic effects of oestrogen and progesterone (Eaton and Eaton, 1999; Eaton *et al*., 1994; Greaves, 2008; Stearns *et al*., 2008; Trevathan *et al*., 2008). It proposes that hormonal contraception in economically advanced societies enhances cyclic exposure to these hormones, and thus increases the rates of breast cancer.

2.6.1. Parity and breast cancer

Several details of the association between parity (number of births) and breast cancer rates restrict the general explanatory power of this hormonal proliferation hypothesis. Perhaps the most important detail is that parity is associated with *increased* breast cancer prior to menopause. This association occurs because rates of breast cancer are elevated during pregnancy and remain substantially elevated for the first year or so after childbirth (Bruzzi *et al*., 1988; Lambe *et al*., 1994; Williams *et al*., 1990). This association restricts the explanatory value of the hormonal proliferation hypothesis to post-menopausal breast cancer (Eaton and Eaton, 1999). Though pre-menopausal breast cancer is not as common as post-menopausal breast cancer, it is generally more rapidly invasive and therefore less amenable to successful treatment. Understanding

pre-menopausal breast cancer is therefore a very important aspect of breast cancer problem.

Post-menopausal proliferation of breast cells is twice as great for nulliparous women as for parous women. However, this difference does not accord well with the hormonal proliferation hypothesis, because women are not experiencing the elevated levels of oestrogen after menopause.

The delay in a protective effect of parity to post-menopausal years, the lack of protective effect of parity prior to menopause and the association of post-menopausal protection only with births soon after puberty has greatly restricted the possible applicability of the hormonal proliferation hypothesis. Specifically to be viable, the hormonal proliferative hypothesis must consider the main oncogenic effects of non-reproductive hormonal cycling to be indirect effects of proliferation during the interval between menarche and the first pregnancy, a period that is extended in economically prosperous countries because menarche occurs at younger ages (Eaton and Eaton, 1999). The hormonal proliferation hypothesis is thus restricted to the indirect effects of proliferation on mutation rather than direct contributions of proliferation to oncogenic proliferation.

2.6.2. Infection and breast cancer

The hormonal proliferation hypothesis was developed without considering the possible involvement of parasitism. Three oncogenic viruses have been associated with breast cancer: EBV, HPV (particularly serotypes 16 and 18), and mouse mammary tumour virus (MMTV; human isolates are generally referred to as MMTV-like) (Table 2.2). Positivity for each virus has been reported for about 25–50% of breast cancers but no more than 10% of the normal breast tissue from the same patients (Bonnet *et al*., 1999; Damin *et al*., 2004; de Villiers *et al*., 2005; Fina *et al*., 2001; Kan *et al*., 2005; Kleer *et al*., 2002; Lawson *et al*., 2001; Wang *et al*., 1995). As is the case for EBV and HPV (see above), MMTV compromises regulation of cell division, apoptosis and cell adhesion (Katz *et al*., 2005; Ouatas *et al*., 2002; effects of MMTV on regulation of telomerase have not yet, to the author's knowledge, been investigated).

If these associations reflect viral causation of breast cancer, they would account for a minimum of about 50% of human breast cancer (i.e., HPV and MMTV must act in conjunction with EBV). Alternatively, if each virus causes breast cancer independently of the others, they could account for nearly 100% of breast cancer (about 50% for EBV and about 25% each for MMTV and HPV). Regardless of this number, evidence implicates oncogenic mutations as well, because oncogenic mutations are found pervasively in breast cancers.

Integration of infectious causation can account for the associations that cannot be explained by the hormonal proliferation hypothesis as well as the paradoxical associations. Rather than restricting the focus to the proliferative effects of reproductive hormones, integration of infectious causation emphasises their suppressive effects on cell-mediated immunity (Doyle *et al.*, 2007, 2008), which may increase vulnerability to persistent viruses. The immune suppression by the infection hypothesis thus explains the elevated risk of breast cancer during pregnancy as a result of effects of the elevated reproductive hormones on the part of the immune system that controls virally infected cells (particularly CD8 + T cells). These effects might occur synergistically with any hormonal enhancement of the proliferation of infected cells, but indirect immunosuppressive effects provide an explanation for the high frequency of premenopausal breast cancers that do not contain receptors for oestrogen or progesterone.

The paradoxical associations with parity can be explained by the effects of mate fidelity and duration of sexual activity on exposure to infection. Women who begin menses earlier will tend to have a longer exposure to sexually transmitted pathogens, such as HPV, and kissing-transmitted pathogens, such as EBV. Having children may reduce exposure to such pathogens because women who are raising families tend to have fewer sexual partners than single women. Moreover, nulliparous women may be nulliparous because they have had a relatively high exposure to the sexually transmitted pathogens that can reduce fertility (as is the case with *Chlamydia trachomatis* and *Neisseria gonorrhoeae*).

2.6.3. The timing of infection and mutations

The hypothesized infectious causation of cancer predicts a temporal pattern of mutations that is not predicted by the hormonal proliferation hypothesis. Specifically, if simultaneous inhibition of the cancer barriers by viruses is needed to initiate oncogenesis, the additional mutations that more completely destroy the barriers to cancer should tend to occur later during oncogenesis. In contrast the hormonal proliferation hypothesis proposes that such mutations are initiating events and should therefore often occur at the onset of oncogenesis.

It is possible to test this prediction. Research on cervical cancer, for example, showed that oncogenic HPV occur at the earliest stages of oncogenesis, before the occurrence of mutations in p53 (Limpaiboon *et al.*, 2000). With regard to breast cancer, mutations in *BRCA* (BReast CAncer susceptibility) and *PTEN* (Phosphatase and TENsin homologue) genes are associated with substantially increased risk. Recent studies have investigated the interactive role of mutations in these genes (Saal *et al.*, 2008). The *PTEN* gene fosters cellular replication and inhibits apoptosis. The *BRCA1*

gene encodes a protein that repairs mutations. Mutations in the *BRCA1* gene therefore contribute to cancer by reducing the repair of mutations in other genes that encode barriers to cancer, and women who inherit a mutant BRCA 1 allele have a higher net rate of mutation in their somatic cells.

Understanding the function of these two genes led researchers to expect that women with the BRCA1 mutation would be susceptible to PTEN mutations, which could then lead the mutated cells down the path to cancer. PTEN mutations do tend to occur in the breast cancers. In contrast to the expectations of researchers studying this association, PTEN mutations occur late during oncogenesis, as expected if viral infection initiates oncogenesis and mutations complete the process after a sufficiently large population of pre-cancerous cells has become established.

2.7. TESTING INFECTIOUS CAUSATION OF CANCER

The breast cancer example illustrates how hypotheses of infectious causation of cancer can be broadly distinguished from hypotheses that rely solely on mutation. Infections are expected at the earliest stage of oncogenesis, but oncogenic mutations are expected later. Because infection-initiated cancers still depend on mutations for cancer progression, evidence that broadly implicates oncogenic mutation (e.g., age-dependent risks of cancer; Frank, 2007) is consistent with infection-initiated oncogenesis. However, despite this consistency, the distinction between mutation-only hypotheses and infection-initiated hypotheses is critical because the infection-initiated hypotheses suggest that cancers can be prevented by preventing infection. The great practical benefit from cancer prevention relative to cancer treatment emphasises the importance of testing the central prediction from the infection-initiated hypothesis, namely that infectious agents tend to be present and oncogenic mutations absent during the earliest phases of oncogenesis.

Most investigations into infectious causation of cancer have attempted to determine whether positivity for the candidate pathogens in cancer tissue is greater than the positivity in unaffected tissue, or whether positivity in cancer patients is greater than in patients without cancer. Ambiguities in interpretation of results can arise for a variety of reasons such as the reliability of assays and evolution of the cancer within a person. One complication occurs because the selective pressures that act within a person are expected to favour loss of the virus. Mutations that fully eliminate the barriers that oncogenic viruses have only compromised will tend to be favoured because the cell with fully eliminated barriers will proliferate faster. Once the barriers are eliminated by mutation, cells

that lose the virus become the most competitive cells of all because they no longer bear the costs of production of viral components and because they are less likely to be destroyed by immune recognition of viral components. Evaluating infectious causation of cancer by testing for the presence or absence of viruses in cancer tissue can therefore be problematic.

Advances in molecular techniques should provide powerful methods for evaluating viral causation of cancer by taking into account these complexities. Molecular phylogenies of cancers may be particularly useful tools for understanding oncogenesis (Abu-Asab *et al.*, 2008). With improvements in the ability to generate nucleic acid and protein sequences from small numbers of cells, the ability to generate molecular phylogenies of the cancer cells within a given patient is becoming increasingly feasible. The predictions that arise from hypotheses of infectious causation can be mapped onto such molecular phylogenies, allowing for rigorous testing of the changes in viral associations with cancers that are predicted from the hypotheses of virally induced oncogenesis (Fig. 2.3).

This approach offers the potential for dramatically reducing the time between the identification of a candidate infectious cause of cancer and the generation of compelling evidence that the pathogen causes the cancer. The general problem is that the old guidelines for acceptance of infectious causation, such as Koch's postulates, cannot be applied effectively for chronic diseases such as human cancers (Cochran *et al.*, 2000). The historical record for acceptance of infectious causation of cancer has

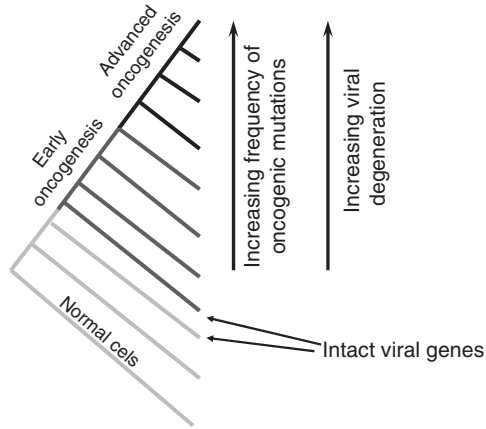

FIGURE 2.3 Predicted cladogram if viruses are a primary cause of cancer. The branches represent different, simultaneously taken samples from one individual (see Abu-Asab *et al.*, 2008 for a discussion of the merits of molecular phylogenies as models for cancer evolution).

shown slow but steady progress over the past three decades (Table 2.1). However, the large number of cancers for which infectious causation is suspected but not yet accepted (Table 2.2) suggests that we are in the midst of the overall process of recognition of the actual role of infectious causation.

The stakes are particularly great because discovery of infectious causation has generally led to some of the greatest public health benefits per unit investment in cancer research. Cancers caused by infection have often been substantially reduced once the infectious agents have been identified; mucosa-associated lymphoid tumour (MALT) stomach cancers have been prevented and even cured with antibiotics (Bayerdörffer *et al.*, 1995), liver cancer has been prevented by screening blood supplies for hepatitis B and C viruses, and, by use of the hepatitis B vaccine, and HPV cancers have been prevented by vaccination. Because cancers are so devastating and treatment of cancers is often both gruelling and marginally effective, the good track record of preventing infection-induced cancers demands that the full spectrum of infectious causation of cancers be investigated and accurately understood.

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CHAPTER 3

Invasion of the Body Snatchers: The Diversity and Evolution of Manipulative Strategies in Host–Parasite Interactions

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Contents

Abstract

Parasite-induced alteration of host behaviour is a widespread transmission strategy among pathogens. Understanding how it works is an exciting challenge from both a mechanistic and an evolutionary perspective. In this review, we use key examples to examine the

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proximate mechanisms by which parasites are known to control the behaviour of their hosts. Special attention is given to the recent developments of post-genomic tools, such as proteomics, for determining the genetic basis of parasitic manipulation. We then discuss two novel perspectives on host manipulation (mafia-like strategy and exploitation of host compensatory responses), arguing that parasite-manipulated behaviours could be the result of compromises between host and parasite strategies. Such compromises may occur when collaborating with the parasite is less costly for the host in terms of fitness than is resisting parasite-induced changes. Therefore, even when changes in host behaviour benefit the parasite, the host may still play some role in the switch in host behaviour. In other words, the host does not always become part of the parasite's extended phenotype. For example, parasites that alter host behaviour appear to induce widely disseminated changes in the hosts' central nervous system, as opposed to targeted attacks on specific neural circuits. In some host–parasite systems, the change in host behaviour appears to require the active participation of the host (e.g., via host immune-neural connections). Even when the change in host behaviour results in clear fitness benefits for the parasite, these behavioural changes may sometimes be produced by the host. Changes in host behaviour that decrease the fitness costs of infection could be selected for, even if these changes also benefit the parasite.

3.1. INTRODUCTION

Animal behaviour is complex. The dance of a honeybee, the dawn chorus of birds, the group hunting of wolves or the cognitive behaviour of tool-using crows and chimps are examples that illustrate this point. Organisms choose where to go, when and where to forage, how and with whom to mate, and whether or not to invest in parental care of the resultant offspring. The study of animal behaviour is a one of the oldest and most established of the natural sciences and remains one of the more accessible arenas of science for the general public through natural history programming. However, the behaviour of parasites is usually neglected. This is not to say that parasites do not behave but only that their behaviour has been considered simple and not particularly interesting. Most parasites are phage or bacteria whose behaviour consists of habitat choice. For multi-cellular parasites such as the liver fluke, *Fasciola hepatica*, a wider range of behaviours exist such as choosing an optimal location, choosing to mate or whether to self-fertilise, and when to reproduce. But it is hardly behaviour on par with the courtship dance of a bower bird.

However, in some host–parasite systems parasites induce complex behavioural changes in their host. For example, some parasitoid wasps

coerce spiders into spinning 'sleeping bags' suspended from branches, thus providing a safe pupating site for the wasp (Eberhard, 2000). Crickets, ants and other insects infected with hairworms (nematomorpha) or nematodes dive into water, allowing the aquatic parasite to exit their body and mate (Maeyama *et al*., 1994; Thomas *et al*., 2002a). Rats infected with *Toxoplasma* develop a fatal attraction for cats, increasing parasitic transmission to their next host (Berdoy *et al*., 2000). These are just some of the many examples of the dramatic behavioural impact parasites can have on their host. Parasites can also have less extreme changes on host behaviour such as shifts in foraging, location or activity (reviewed in Moore, 2002). If the change in host behaviour benefits the parasite (e.g., by increasing transmission to a new host) we suggest that the parasite has been selected to produce this behavioural change in its host. In this case, the altered behaviour of the host is a phenotype of the parasite and is controlled by the parasite's genes. Therefore, it is part of the parasite's extended phenotype.

This concept of the extended phenotype was first developed by Richard Dawkins (1982). In his book, *The Extended Phenotype*, he argues that a gene can produce a phenotype that extends beyond the body of a single individual, if such a phenotype results in increased transmission of the gene to the next generation. A phenotype is usually defined as a trait of the individual organism such as eye or flower colour. The extended phenotype perspective includes abiotic structures such as birds' nests. Birds' nests increase the fitness of bird genes; therefore, a nest is an example of an extended phenotype. Parasitic manipulation of host behaviour leading to increased transmission of the parasite's genes is another example of an extended phenotype (Dawkins, 1982, 1990, 2004).

The extended phenotype perspective is not a theory, but is merely a 'way of viewing the facts'. We know that the presence of parasites alters the behaviour of their hosts in ways that range from simple to dramatic. Behaviour is a phenotype and has a genetic basis. In infected hosts either parasite genes or host genes are responsible for its altered behaviour. The extended phenotype perspective merely postulates that in some host–parasite interactions the parasite genes are responsible for the aberrant behaviour. This parasite-centred view has been relatively ignored in evolutionary ecology (Poulin, 2007), sometimes for good reason (Thomas *et al*., 2005, see Box 3.1).

In this review we will examine the mechanisms by which parasites are known to control the behaviour of their hosts. Despite years of study, we lack unequivocal evidence that parasite genes cause host behavioural change. Thus we advocate that future studies should use a more sophisticated approach than has been previously adopted (e.g., the incorporation of more molecular techniques for determining the genetic basis of manipulation). We review the advances that have been made in the field using proteomic tools. We also explore the degree to which host behavioural changes could be a compromise between host and parasite strategies.

BOX 3.1 Brief history of parasitic manipulation

Cram (1931) and **Van Dobben (1952)** first suspected that parasites might have the ability to modify the behaviour of their hosts in a way that increases their transmission efficiency. The pioneering works of Bethel and Holmes on acanthocephalan worms (1973, 1974, 1977) significantly advanced this hypothesis. However, it was only with the publication of Richard Dawkins' book entitled '*The Extended Phenotype*' (1982, see main text for details) that the field of parasitic manipulation acquired a conceptual framework. Henceforth, parasitologists considered that host alteration may be regarded as the expression of the genes of the parasite in the host phenotype and that some of the parasite's genes are selected for their effect on host phenotype.

Dawkins' way to view facts has led researchers to consider all behavioural changes observed in an infected organism as beneficial for the parasite. However, not all parasite-induced alterations of the host phenotype necessarily enhance parasite transmission. Some alterations can be adaptations of the hosts to defend themselves against parasites (e.g., behavioural fever, Moore, 2002). Moreover, changes might be pathological consequences of infection, adaptive to neither host nor parasite. These are termed 'boring by-products' of infection (Dawkins, 1990; Edelaar *et al*., 2003; Webster *et al*., 2000).

Robert Poulin (1995) wrote an important paper that helped address the issue of adaptive versus non-adaptive host behavioural changes by highlighting the need for a clear approach to interpreting potential cases of parasitic manipulation (Poulin, 1995). Four criteria were proposed in order to consider changes as adaptive for the parasite in the context of transmission: complexity, purposiveness of design (i.e., conformity between *a priori* design and the host phenotypic alterations), convergence (similar changes in several independent lineages) and fitness consequences. Poulin's (1995) paper marked the start of a new period during which many studies taking into account these recommendations appeared in the scientific literature. It also marked a departure from purely adaptationist reasoning.

This paper has nonetheless left one point obscure: should we consider the changes that are pathological consequences of infection *and* are coincidentally beneficial for the parasite as adaptations? In his paper, Poulin (1995) distinguished between 'true' parasite manipulation and 'by-products' of infection (the latter being changes coincidentally beneficial that may be a fortuitous payoff of other adaptations). This point faced criticism since it is almost impossible to distinguish between the primary focus of historical selection and concomitant effects on transmission (see Lefèvre and Thomas, 2008; Moore, 2002 and Thomas *et al*., 2005 for details).

Two scenarios of parasitic manipulation are presented (i.e., the exploitation of host compensatory responses and 'mafia-like manipulation') in which the parasite-manipulated behaviours are not necessarily an illustration of the extended phenotype and can benefit both partners.

As has been common in the field of parasitic manipulation of host behaviour, our discussion will span multiple fields. For parasitologists we aim to provide key information regarding parasite-mediated activities; for behavioural ecologists, whose focus is behaviour, we want to highlight the myriad forms of manipulation and how we are beginning to understand the proximate mechanisms underlying them; for evolutionary biologists, who are interested in trait evolution and co-evolutionary processes, we want to review this exciting field for them; and for applied scientists who either deal with human or veterinary diseases or use parasites as biocontrol, we want to emphasise that behavioural studies are highly relevant to applied fields.

3.2. HOW PARASITES ALTER HOST BEHAVIOUR

Most research on parasitic manipulation of behaviour has focused on the effects of parasites on host neural function. This emphasis is reasonable given that behaviour is controlled by the central nervous system (CNS). Below we review two examples in which parasites are known to alter the neural function of their host (for more examples see Adamo, 1997, 2002; Klein, 2003; Moore, 2002; Thomas *et al*., 2005). These examples illustrate that the mechanisms mediating host behavioural change are often complex. Parasites do not manipulate the brain of their hosts the way a puppeteer controls a puppet, delicately tweaking only those neural circuits responsible for specific behaviours. Instead parasites appear to slug the host's brain with a number of diffuse and widespread effects, some of which induce changes in host behaviour. We continue by showing how post-genomic era approaches can lead to great advances in our understanding of the proximate mechanisms mediating host behavioural change. In particular we discuss the recent parasito-proteomics studies of infected host brains. We end with a discussion of the importance of this new technique, especially in light of the complex mechanisms that are typically involved in host behavioural change.

3.2.1. Parasitic effects on host neural function

3.2.1.1. Rabies

Rabies is often cited as a classic example of parasitic manipulation of host behaviour (Klein, 2005). As in other cases of parasitic manipulation, the parasite is thought to commandeer the neural circuits that regulate

specific host behaviours. Changing these specific host behaviours benefits the parasite. Below we examine the evidence for this scenario.

Rabies is caused by RNA viruses of the genus *Lyssavirus* (Rupprecht *et al.*, 2002). Rabies virus infects the CNS of its host and induces profound behavioural changes (Rupprecht *et al.*, 2002). Some of these behavioural changes (e.g., aggressiveness and hyper-salivation) increase viral transmission (Hemachudha *et al.*, 2002). The rabies virus docks with specific neural receptors suggesting specificity in its attack on the host's CNS (Hemachudha *et al.*, 2002). Once inside a neuron, the rabies virus alters ion homeostasis and synaptic physiology, both of which alter neural transmission (Dhingra *et al.*, 2007). This effect may explain why neural transmission is abnormal in some brain regions in rabies (Fu and Jackson, 2005). Interestingly, changes also occur in neurons that do not appear to be directly infected with the virus, suggesting that the virus can also influence neural function indirectly (Fu and Jackson, 2007). Neuronal damage is minimal during the period in which an infected animal is transmitting the virus (i.e., prior to severe motor symptoms, Scott *et al.*, 2008). Therefore, the virus has the tools to selectively alter host behaviour by manipulating specific target neurons without killing them.

Nevertheless, the rabies virus does not selectively alter either behaviour or neural function. For example, rabies virus induces more than just aggression and hyper-salivation in its host. Infected hosts also suffer from a lack of appetite and have reduced co-ordination (Ruppecht *et al.*, 2002). These behaviours are unlikely to enhance viral transmission and demonstrate that the effects of rabies are not entirely selective. Although non-specific effects might be expected from any virus that infects the brain, behaviours that are important for enhanced transmission (e.g., increased aggression) would be expected to occur in all hosts of a manipulative parasite. However, not all animals infected with rabies are aggressive (Hemachudha *et al.*, 2002). Most rabies victims can be divided into two groups based on their behavioural symptoms: encephalitic (furious) and paralytic (dumb) (Hemachudha *et al.*, 2002). Aggressive behaviour is observed only in encephalitic rabies. In paralytic rabies, the host gradually loses motor control and consciousness (Hemachuda *et al.*, 2003). Although these symptoms would increase the host's contact with predators, this is unlikely to lead to increased viral transmission because the rabies virus is fragile and non-bite transmission of rabies (e.g., via mucous membranes) is rare (Rupprecht *et al.*, 2002). In paralytic rabies, the lack of aggression coupled with the animal's decreased mobility and increased lethargy probably results in reduced viral transmission. Nonetheless, paralytic rabies is not a rare form and about 25% of infected humans have paralytic rabies (Hemachudha *et al.*, 2002). The paralytic form of rabies is also common in dogs (Laothamatas *et al.*, 2008), even though dogs are the co-evolved host for the canine variant of the virus

(Hemachudha *et al*., 2003). Differences in the genetic code of the virus are not responsible for the differences in the behaviour of infected hosts (Hemachudha *et al*., 2003). For example, Hemachudha *et al.* (2002) report a case in which the same rabid dog induced paralytic rabies in one victim and encephalitic rabies in the other. Therefore, the rabies virus induces aggression in only some of its hosts, despite the likely importance of this behaviour for viral transmission. Moreover, the virus also induces other behaviours in its host that probably impede viral transmission.

The rabies virus does not selectively target those brains areas responsible for regulating aggression (Laothamatas *et al*., 2008). For example, in humans, the amygdala (a part of the limbic system) and the orbitofrontal cortex regulate aggression (Coccaro *et al*., 2007). Although the virus reliably strikes the limbic system, the virus does not infect these structures exclusively, or even preferentially (Laothamatas *et al*., 2003). During rabies in humans, magnetic resonance imaging (MRI) shows changes in brainstem, cerebellum, hippocampi (and other parts of the limbic system, including the amygdala), hypothalamai, deep and sub-cortical white matter, and deep and cortical gray matter (Laothamatas *et al*., 2003). The amygdala is thought to be critical for the regulation of aggression in non-human mammals too (Kandal *et al*., 1991). Nevertheless, the rabies virus does not target the amygdala in non-human hosts either. For example, rabid dogs have high concentrations of rabies viral messenger RNA (mRNA) in the basal ganglia, caudate nucleus, cerebellum, hippocampus (and other parts of the limbic system), medulla, mid-brain, pons, thalamus and the frontal, parietal, occipital and temporal lobes of the cerebrum (Laothamatas *et al*., 2008). Interestingly, the distribution of virus in the brain is the same in both paralytic and encephalitic forms of rabies (Laothamatas *et al*., 2003, 2008; Smart and Charlton, 1992). The limbic system is attacked in both forms, but only results in increased aggression in encephalitic rabies.

The evidence above demonstrates that the simplest postulated mechanism of manipulation—that is, that the rabies virus selectively infects and manipulates those brain areas that regulate aggressive behaviour in mammals—is false. How then does rabies produce enhanced aggression in a large portion of its hosts? Hemachudha *et al.* (2002) suggest that the immunological reactions provoked by the virus (see Hooper, 2005) play a role in changing host behaviour. For example, cytokines, released during the body's response to the rabies virus, could alter limbic system function (Hemachudha *et al*., 2002) and this may increase aggression. Increases in some cytokines can increase aggressiveness (Kraus *et al*., 2003), supporting this hypothesis. In fact, immune reactions alone can produce aggressive behaviour. For example, during auto-immune disorders such as paraneoplastic limbic encephalitis, the immune system damages the limbic system (Osborne, 1994) and this induces aggressive

behaviour in some patients (Tardiff, 1998). Therefore, the effect of the virus on host behaviour may depend on the host's immune response (Hemachudha *et al.*, 2002), and this would explain why the effects of the virus on host behaviour are variable. However, Charlton *et al.* (1984) found no significant change in the aggressiveness of rabid skunks given the immunosuppressant cyclophosphamide compared to controls (Charlton *et al.*, 1984). Rabies virus replication was increased by cyclophosphamide treatment (i.e., brains of treated animals had higher viral titres), demonstrating that cyclophosphamide did suppress host immune responses (Charlton *et al.*, 1984). Recently, Laothamatas *et al.* (2008) found that dogs with paralytic rabies had a more robust immune response to the virus and showed greater cytokine release in all brain areas (including the limbic system) than dogs with encephalitic rabies, the opposite to what would be predicted if cytokines are driving the increase in aggression. Moreover, MRI studies revealed greater brain abnormalities in the non-aggressive paralytic dogs than in encephalitic dogs, even though dogs with paralytic rabies have less viral mRNA expressed in their brains compared to dogs with encephalitic rabies (Laothamatas *et al.*, 2008). These results suggest that the robust immune response of some animals may prevent the virus from altering host behaviour, leading to the paralytic form of rabies. Further studies are required to clarify the role of the host's immune system in the production of aggressive behaviour during rabies.

The Borna disease virus (BDV) is another virus of the CNS that induces aggressive behaviour in its host (Klein, 2003). However, similar to rabies, not all infected animals show an increase in aggressive behaviour (Carbone *et al.*, 1987). As with rabies, BDV infects multiple brain areas, including the limbic system (see Klein, 2003). In BDV infections, the virus replicates first in the hippocampus (Carbone *et al.*, 1987). However, by the time behavioural symptoms such as aggressive behaviour occur, the virus has widely disseminated throughout the brain (Carbone *et al.*, 1987). Moreover, the behavioural symptoms occur at the same time that the host's immune response produces widespread inflammation in the brain (Carbone *et al.*, 1987). Therefore, the immune system may play a role in inducing host aggression in both rabies and BDV.

3.2.1.2. Gammarids, parasites and serotonin

Unravelling the connections between parasites, neural transmission and altered host behaviour may be easier to discover when the host is an invertebrate rather than a vertebrate host (Helluy and Holmes, 2005). In this section we review the mechanisms used by different parasites to alter the behaviour of small crustaceans known as gammarids. Gammarids are attacked by parasites that often have complex life cycles in which the parasite requires transmission to a vertebrate host to complete its

development (e.g., Kennedy, 2006). In some of these systems, once the parasite reaches the infective stage, the parasitised host shows changes in escape behaviour resulting in an increased likelihood that the infected gammarid will be consumed by the parasite's appropriate vertebrate host. Some of these changes probably occur because of parasite-induced changes in the host's serotonergic neural signalling system (Table 3.1).

For example, when the acanthocephalan *Polymorphus paradoxus* reaches the infective stage, its gammarid host, *Gammarus lacustris*, changes its escape behaviour. The parasitised host swims towards the light and clings to the nearest solid material when disturbed, instead of swimming away from the light and burrowing into the mud as non-parasitised controls do. Some of the same behaviours induced by the presence of the parasites can be mimicked by injections of serotonin. Injections of other biogenic amines, such as octopamine or dopamine, do

TABLE 3.1 Relationship between presence of the parasite, increased host phototaxis and serotonin immunohistochemistry

Gammarid (Host)	Parasite	Effect of parasite on phototaxis	Effect of serotonin on phototaxis	Effect of parasite on serotonin staining of the CNS[a]
Gammarus insensibilis	*Microphallus papillorobustus* (within CNS)	Increase	Increase[b]	Decrease TGN smaller
Gammarus lacustris	*Polymorphus paradoxus* (within haemocoel)	Increase	Increase	Increase in varicosities
Gammarus pulex	*Pomphorhynchus laevis* (within haemocoel)	Increase	Increase	Increase
	Polymorphus minutus (within haemocoel)	None	Increase	None
Gammarus roeseli	*Pomphorhynchus laevis* (within haemocoel)	None	Increase	None

[a] The study on *G. lacustris* examined only the ventral nerve cord. All other studies examined staining in the cerebral ganglion
[b] cited in Helluy and Thomas (2003); TGN, tritocerebral giant neuron.
Notes: See text for references.

not induce these behaviours. Serotonin haemolymph concentrations need to be raised by approximately three orders of magnitude above physiological levels to produce the effect. The need for such large concentrations may suggest that serotonin is acting through the CNS and not via peripheral receptors (Helluy and Holmes, 1990).

Immunohistochemical staining for serotonin reveals that infected animals have the same number of serotonergic cells as controls. However, the fine structure of the neurons differs in infected animals. Infected individuals have an apparent increase in the serotonergic staining of structures thought to be axon terminals (Maynard *et al.*, 1996). This result could signal a change in the amount of serotonin released into the synapse. For example, reduced serotonin release would result in a build up of serotonin within the axon terminal of the neuron, creating the increase in staining. Regardless of whether serotonin release is increased or decreased, Maynard *et al.*'s (1996) results suggest that the parasite has an impact on the host's serotonergic system. How the parasite exerts this effect is unknown.

A related gammarid, *Gammarus pulex*, is infected with the acanthocephalans *Polymorphus minutus* and *Pomphorhynchus laevis*. Hosts infected with *P. minutus* show a reversed geotaxis compared to control animals and swim towards the surface. This change in behaviour probably increases the chance that the host comes in contact with its definitive host, a bird. *P. minutus* does not induce phototaxis. *P. laevis*, however, changes the photophobia of *G. pulex* into phototaxis, resulting in the host swimming towards the light. This behaviour makes the host more vulnerable to fish predation, the definitive host for this species. Injections of serotonin induce phototactic behaviour but do not change geotactic behaviour in *G. pulex*. As in *G. lacustris*, large doses of serotonin are needed to induce the effect (Tain *et al.*, 2006).

Immunohistochemical staining of the cerebral ganglion for serotonin shows no gross differences between hosts infected with either parasite and uninfected controls (e.g., in the number of serotonergic cells). Tain *et al.* (2006) also found no difference in the gross anatomy of the giant serotonergic neuron found in the brain (i.e., the tritocerebral giant neuron (TGN)) in infected animals. However, hosts infected with *P. laevis* had enhanced immunohistochemical staining for serotonin, but there was no difference in the intensity of staining when they were infected with *P. minutus*. Therefore, staining for serotonin only increased when host phototactic behaviour increased (Tain *et al.*, 2006).

A related gammarid (*Gammarus roeseli*) is also infected with the parasite *P. laevis*. However, in this gammarid, *P. laevis* does not induce phototactic behaviour, even though injections of serotonin can induce phototaxis in *G. roeseli*. *G. roeseli* shows no change in serotonergic staining when infected (Tain *et al.*, 2007), supporting the hypothesis that altered serotonin

signalling is causally involved in the change in host phototactic behaviour (Table 3.1).

The gammarid *Gammarus insensibilis* is parasitised by the trematode *Microphallus papillorobustus*. In the previous examples, the parasites remain outside of the CNS (Kennedy, 2006). In this system, however, the trematode lodges within the host's protocerebrum, a part of the cerebral ganglion and CNS. The host is not debilitated, but instead shows altered responses to specific sensory stimuli such as light. Once the parasite reaches the infective stage, the host shows aberrant escape behaviours making it more likely to be consumed by the parasite's definitive host, a bird (Helluy and Thomas, 2003).

Immunohistochemical staining for serotonin reveals profound changes between infected and control animals. For example, the TGN is stunted in infected animals suggesting some degeneration of serotonergic fibres. Such a change in neural architecture is very likely to produce decreases in serotonergic signalling within the CNS because of likely decreases in the synaptic field. However, some parts of the brain showed no change in serotonergic staining, suggesting that the effect was specific to certain neurons or brain areas. The parasite does not appear to influence the TGN by mechanically squeezing it; the giant neuron and the parasite reside on opposite sides of the brain (Helluy and Thomas, 2003). How the parasite alters the morphology, and presumably the function, of this serotonergic neuron remains unknown. Unfortunately the role the TGN plays in the host's escape behaviour is also unknown.

Taken together, the results (Table 3.1) suggest that parasites can influence gammarid phototactic behaviour by altering some aspect of the serotonergic system. However, the results are puzzling because it is unclear whether an increase or decrease in serotonin release within the CNS is responsible for altering phototactic behaviour. Immunohistochemical studies do not provide a reliable estimate of neural activity or neurotransmitter release (see de Jong-Brink and Koene, 2005) especially when looking across different physiological states (e.g., parasitised vs non-parasitised, see Zitnan *et al*., 1995).

The observation that injections of serotonin into the haemocoel induce increased phototaxis suggests that enhanced serotonergic release is responsible for the increase in phototaxis. However, all the parasites except *M. papillorobustus* remain outside the host's CNS (Kennedy, 2006). It is unlikely that the parasites residing in the haemocoel can produce enough serotonin to induce phototaxis (Holmes and Zohar, 1990; Tain *et al*., 2006; Thomas *et al*., 2005). Most likely the parasites induce the host's CNS to produce serotonin. Ponton *et al*. (2006a) found that one of the enzymes important for serotonin production, aromatic amino acid decarboxylase (Cooper *et al*., 2002), was not visible on two-dimensional (2D) electrophoresis gels of the brains of *G. pulex* parasitised with

P. minutus, but was visible on gels of uninfected brains. Ponton *et al.* (2006a) interpreted their results as indirect evidence of an increase in aromatic acid decarboxylase activity. However, these data are equivocal, and like immunohistochemical staining, can also support the hypothesis that serotonin production has declined. Moreover, in vertebrates the decarboxylation step is not the rate-limiting step in the synthesis of serotonin; the rate-limiting step is governed by tryptophan hydroxylase and the availability of tryptophan (Cooper *et al.*, 2002). Given that the amount of *L*-tryptophan in the crayfish brain is typically more than five times greater than that of 5-hydroxytryptophan (Rodriguez-Sosa *et al.*, 1997) the situation is probably similar in crustaceans. Therefore if serotonin production is increased in parasitised brains, there should be a concomitant increase in tryptophan and tryptophan hydroxylase concentrations. More direct measurements of serotonin synthesis in parasitised brain tissue are needed (e.g., using high-performance liquid chromatography (HPLC)). Pharmacological (e.g., Tierney *et al.*, 2004) and electrophysiological studies would also be helpful in determining how altered serotonergic activity is related to the increase in phototaxis.

As with rabies, the mechanisms mediating behavioural manipulation of infected gammarids are complex. For example, host immune responses may also play a role in producing the change in host behaviour (Tain *et al.*, 2007). As in rabies, the manipulative parasites (e.g., *P. laevis*, Cézilly and Perrot-Minnot, 2005) induce other behavioural changes in their host, in addition to phototaxis. This effect may reflect the fact that serotonin signalling is involved in many behaviours in crustaceans (e.g., see Weigner, 1997). Most of the studies in Table 3.1 suggest widespread alterations in the functioning of the serotonergic system, not a selective strike on specific neural circuits.

3.2.2. Proteomics and proximate mechanisms

Post-genomic technology promises to revolutionise many fields in biology by providing enormous amounts of genetic data from non-model organisms. Proteomics is a case in point and promises to bridge the gap between our understanding of genome sequences and cellular behaviour; it can be viewed as a biological assay or tool for determining gene function (for explanations of genomic terms see Box 3.2). Parasito-proteomics is the study of the reaction of the host and parasite genomes through the expression of the host and parasite proteomes (genome-operating systems) during their complex biochemical cross-talk (Biron *et al.*, 2005a,b). Proteomics, with the ability to investigate the translation of genomic information, offers an approach to study the global changes in protein expression of the host CNS caused by parasites. Fig. 3.1 outlines the essential steps to any proteomics study of parasite manipulation of host behaviour.

BOX 3.2 Glossary for the 'omics' tools use in parasite-proteomics

Genome: The full complement of genes carried by a single (haploid) set of chromosomes. The term may be applied to the genetic information carried by an individual or to the range of genes found in a given species.

Genomics: It is the study of an organism's genome and the use of the genes. It deals with the systematic use of genome information, associated with other data, to provide answers in biology, medicine and industry.

Immunochemistry: A branch of chemistry that involves the study of the reactions and components on the immune system. Various methods in immunochemistry have been used in scientific study, from virology to molecular evolution.

Interactome: The interactome is the whole set of molecular interactions in cells. It is usually displayed as a directed graph. When spoken in terms of proteomics, it refers to protein–protein interaction network (PPI) or protein network (PN).

Gene knock-out: This is a genetic technique in which an organism is engineered to carry genes that have been made inoperative. Gene knock-in is similar to knock out, but instead it replaces a gene with another instead of deleting it.

Neuropeptidome: In recent years, the introduction of highly sensitive mass spectrometry paved the way for rapid screening of the neuropeptide profile (neuropeptidome) even to the single cell level, in species as small as insects.

Neuropeptides: Neuropeptides are the most structurally diverse messenger molecules that influence a wide range of physiological processes. They are present in all Metazoa that have developed a nervous system.

Proteome: The term proteome was first used in 1995 and has been applied to several different types of biological systems. A cellular proteome is the collection of proteins found in a particular cell type under a particular set of environmental conditions such as exposure to hormone stimulation. It can also be useful to consider an organism's complete proteome. The complete proteome for an organism can be conceptualised as the complete set of proteins from all of the various cellular proteomes. This is very roughly the protein equivalent of the genome. The term 'proteome' has also been used to refer to the collection of proteins in certain sub-cellular biological systems. For example, all of the proteins in a virus can be called a viral proteome.

(*continued*)

BOX 3.2 (*continued*)

Proteomics: The large-scale study of proteins, particularly their structures and functions. This term was used to make an analogy with genomics, and is often viewed as the 'next step' but proteomics is much more complicated than genomics.

RNAi: Small fragments of double-stranded RNA whose sequence matches the transcribed sequence of a gene. This technique is used to decrease the expression of a gene by disabling the transcribed mRNA.

Transcriptome: Is the whole set of mRNA species in one or a population of cells.

Transcriptomics: Techniques to identify mRNA from actively transcribed genes.

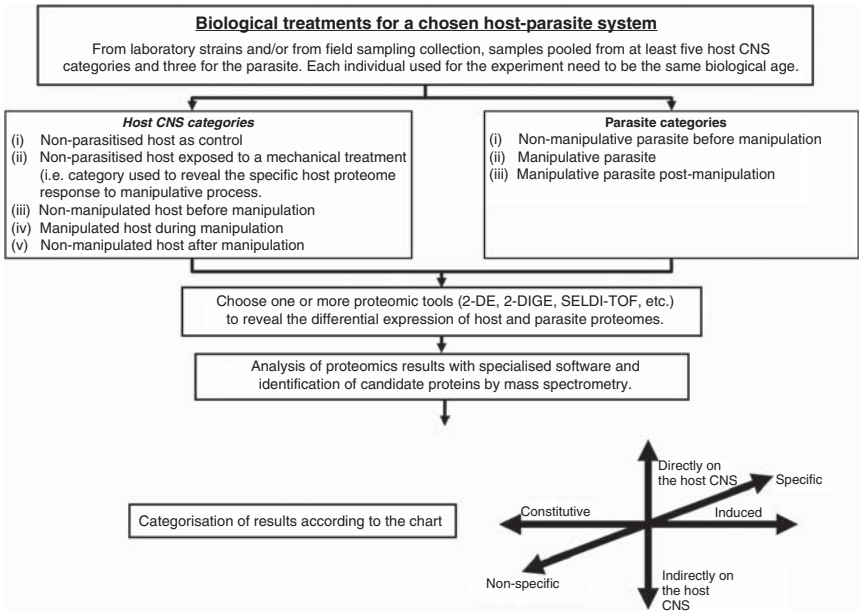

FIGURE 3.1 Flowchart for the study of manipulative strategies with parasito-proteomics. CNS, central nervous system; 2-DE, two-dimensional gel electrophoresis; 2D-DIGE, two-dimensional-difference gel electrophoresis; SELDI-TOF, surface enhanced laser desorption/ionization time-of-flight.

3.2.2.1. Pioneer parasito-proteomics studies on parasitic manipulation

Pioneer proteomics studies have been carried out on six arthropod host–parasite systems: two orthoptera–hairworm systems, two insect vector–pathogen systems and two gammarid–parasite systems. Table 3.2 summarises the proteomics tools used and the proteome responses for the

TABLE 3.2 Synopsis of 'parasito-proteomics' studies on parasitic manipulation

Host–parasite association		Proteomics tools			Proteome response				
					In head host		In parasite		
Host species	*Parasite species*	Separation of proteins	IP scale; Mw scale	Identification of proteins	TNSA	PPSLMP	TNSA	*PPSLMP*	References
Nemobius sylvestris (Bosc) (Orthoptera, Gryllidae)	*Paragordius triscupidatus* (Dufour) (Nematomorpha, Gordiidae)	2-DE	pH 5–8; 19–122 kDa	MS, MS/MS, Sequencer	902	3.8	729	5.0	Biron *et al.*, 2006
Meconema thalassinum (De Geer) (Orthoptera, Tettigoniidae)	*Spinochordodes tellinii* (Nematomorpha, Spinochordodidae)	2-DE	pH 5–8; 19–122 kDa	MS	566	16.8	763	5.0	Biron *et al.*, 2005
Anopheles gambiae (Giles) (Diptera, Culicidae)	*Plasmodium berghei* (Haemosporida, Plasmodiidae)	DIGE	pH 3–10; 14–100 kDa	MS, MS/MS	1400	0.9	No data	No data	Lefèvre *et al.*, 2007a
Glossina palpalis gambiensis (Diptera, Glossinidae)	*Trypanosoma brucei brucei* (Kinetoplastida, Trypanosomatidae)	2-DE	pH 3–10; 20–122 kDa	MS	816	2.9	No data	No data	Lefèvre *et al.*, 2007b
Gammarus insensibilis (Amphipoda, Gammardiae)	*Microphallus papillorobustus* (Trematoda, Microphallidae)	2-DE	pH 3–6; 20–122 kDa	MS	556	12.9	No data	No data	Ponton *et al.*, 2006a
Gammarus pulex (Amphipoda, Gammardiae)	*Polymorphus minutus* (Acanthocephala, Polymorphidae)	2-DE	pH 3–6; 20–122 kDa	MS	838	8.1	No data	No data	Ponton *et al.*, 2006a

Notes: 2-DE, two-dimensional gel electrophoresis; DIGE, difference gel electrophoresis; IP, isoelectric point; Mw, molecular weight; TNSA, total number of proteins spots analysed; PPSLMP, percentage of protein spots potentially linked to manipulative process; MS, mass spectrometry.

host CNS of each host–parasite association. In each study, multiple treatments were carried out to control for potential confounding effects and to exclude the proteins that are non-specific to the manipulative process making it easier to find the proteins potentially linked with host behavioural changes.

Initially, proteomics was used to explore the mechanisms in host CNS underlying the suicidal behaviour of crickets and grasshoppers when manipulated by their hairworms (Biron *et al.*, 2005c, 2006). Two orthoptera-hairworm systems have been investigated: (i) the cricket, *Nemobius sylvestris*, parasitised by the hairworm, *Paragordius tricuspidatus*; (ii) the long-horned grasshopper, *Meconema thalassinum*, parasitised by the hairworm, *Spinochordodes tellinii* (details on the background biology can be found in Thomas *et al.*, 2002a). Because hairworm parasites are very big (i.e., worm length exceeds that of the host by 3–4 times) and because they are located in the body cavity, it is very easy to separate the host CNS and the parasite, thereby allowing the simultaneous study of both proteomes without the risk of contamination.

Proteomics studies suggest that adult hairworms produce host mimetic proteins and manipulate behaviour with them. These proteins are from the Wnt family suggesting a direct action of the hairworms on the host's CNS that can lead directly to an alteration of the host behaviour or indirectly via a host genome response. The analysis of the head proteomes revealed that the percentage of proteins potentially linked to the hairworm manipulative process is higher for *M. thalassinum* compared to *N. sylvestris* (see Table 3.2) (Biron *et al.*, 2006). For the hairworms, some of the proteins potentially linked to the manipulative process are the same (see Table 3.2). The altered functions are similar for both orthopteran species except for some families of proteins that are involved in geotactic behaviour, in protein biosynthesis and in recovery following an infection being only differentially expressed in *M. thalassinum* (Fig. 3.2). In the brain of manipulated orthoptera, differential expression of proteins specifically linked to neurogenesis, the visual process, the geotactic process, and neurotransmitter activities have been observed (Fig. 3.2). The altered physiological compartments are similar for both nematomorph species except for some families of proteins implicated in endopeptidase inhibition, in protein folding and in transcriptional regulation that are only expressed in *S. tellinii* (Fig. 3.3; Biron *et al.*, 2006).

Insect vectors (e.g., mosquitoes carrying malaria) are often manipulated to increase encounter rates with vertebrate hosts in ways that enhance the pathogen's transmission (Hurd, 2003; Lefèvre and Thomas, 2008; Lefèvre *et al.*, 2006; Moore, 1993; Rogers and Bates, 2007). Two parasito-proteomics studies have been performed on such systems: (i) *Anopheles gambiae-Plasmodium berghei* (Lefèvre *et al.*, 2007a); (ii) *Glossina papalis gambiensis-Trypanosoma brucei brucei* (Lefèvre *et al.*, 2007b).

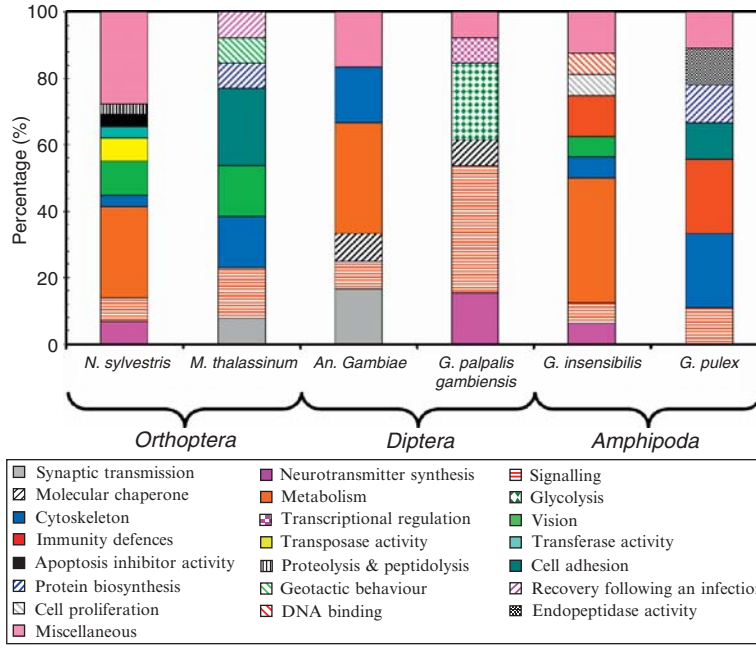

FIGURE 3.2 Proportion of identified proteins linked to a biological process and differentially expressed during the manipulative process in head proteomes of the arthropod hosts for six host–parasite systems. (i) the cricket, Nemobius sylvestris, parasitised by the hairworm, Paragordius tricuspidatus; (ii) the long-horned grasshopper, Meconema thalassinum parasitised by the hairworm, *Spinochordodes tellinii*; (iii) Anopheles gambiae-Plasmodium berghei; (iv) Glossina papalis gambiensis-Trypanosoma brucei brucei; (v) Gammarus insensibilis parasitised by the trematode, Microphallus papillorobustus; (vi) Gammarus pulex parasitised by the ancantocephalan, Polymorphus minutes.

These studies provide evidence that the pathogens can alter the head proteome of their insect vectors (see Table 3.2; Fig. 3.2). Some of the altered protein families are similar between dipterans (i.e., sugar metabolism, signal transduction and heat shock response) (see Fig. 3.2). An alteration in energy metabolism has been observed in the CNS of both parasitised hosts (Lefèvre *et al.*, 2007a,b). Finally, these parasito-proteomics studies suggest that *P. berghei* and *T. b. brucei* can alter host apoptosis pathways and sugar metabolisms.

Several parasites such as trematodes, cestodes and acanthocephalans alter the behaviour of their intermediate host to enhance trophic transmission (Moore, 2002; Thomas *et al.*, 2005). To date we have proteomes of two Amphipoda-parasite systems that were also discussed in Section 3.2.1.2: (i) *Gammarus insensibilis* parasitised by the trematode, *Microphallus*

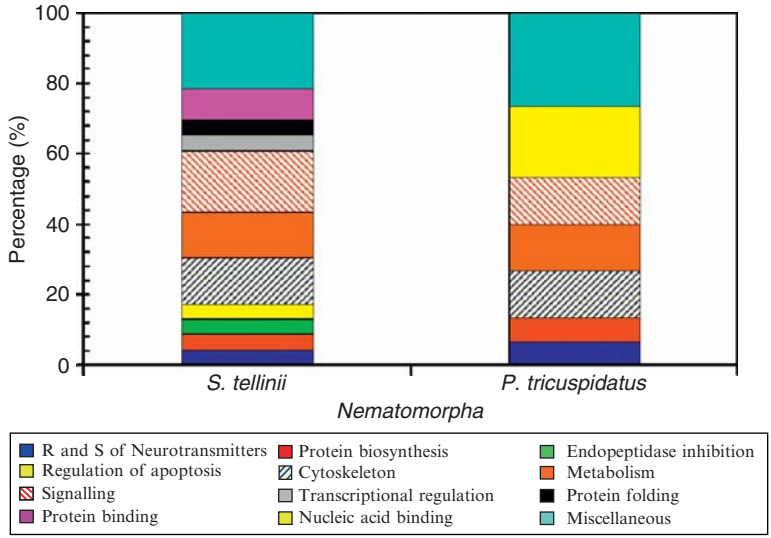

FIGURE 3.3 Proportion of identified proteins linked to a biological process and differentially expressed during the manipulative process in proteomes of two nematomorph species for two orthoptera-hairworm systems. (i) the cricket, Nemobius sylvestris, parasitised by the hairworm, Paragordius tricuspidatus; (ii) the long-horned grasshopper, Meconema thalassinum parasitised by the hairworm, *Spinochordodes tellinii*.

papillorobustus; (ii) *Gammarus pulex* parasitised by the acanthocephalan, *Polymorphus minutes* (Table 3.2). *M. papillorobustus* has a complex life cycle, including snails as first intermediate hosts, gammarids as second intermediate hosts and various sea- and shorebirds as definitive hosts. The life cycle of *P. minutus* displays broad ecological similarities with *M. papillorobustus* since it also involves a gammarid as intermediate host and aquatic birds (mainly ducks) as definitive hosts. Metacercariae of *M. papillorobustus* are always encysted in the brain of *G. insensibilis*, while cystacanths of *P. minutus* are located in the body cavity of *G. pulex*. Both parasites manipulate the behaviour of their gammarid intermediate host, making them more likely to be eaten by predatory definitive hosts at the water surface. *M. papillorobustus* induces a positive phototaxis and a negative geotaxis to alter the behaviour of its intermediate hosts while *P. minutus* induces only a negative geotaxis (Cézilly *et al.*, 2000; Helluy, 1984).

For the two gammarid species, the proteome of *G. insensibilis* displayed a slightly stronger response to the manipulative process caused by its trematode compared to *G. pulex* manipulated by its acanthocephalan (see Table 3.2, Fig. 3.2). The altered functions are similar for both gammarid species except for some families of proteins only expressed in *G. insensibilis*:

those involved in visual process, DNA binding, cell proliferation and metabolism. The proteomic results (Ponton *et al.*, 2006a) obtained for *G. insensibilis–M. papillorobustus* corroborated previous studies suggesting a major role of serotonin in the expression of the aberrant evasive behaviour (see Section 3.2.1.2).

It has been suggested that immune responses may secondarily affect host nervous system functions and hence behaviour and it is increasingly suggested that parasites could exploit host defence reactions in order to manipulate host behaviour (see above; Adamo, 2002; Thomas *et al.*, 2005). The proteomics results have shown that arginine kinase is differentially expressed in the brain of infected *G. insensibilis* and *G. pulex* compared to uninfected individuals. This phosphotransferase is known to be one of the regulating factors in nitric oxide (NO) synthesis (Mori and Gotoh, 2000). NO is liberated during immunological reactions, but it also acts as a neuromodulator. Thus, these proteomic results provide supportive evidence for the hypothesis suggesting that parasites could exploit host defence reactions in order to manipulate host behaviour.

3.2.2.2. Parasito-proteomics and parasite manipulation: A bright future?

Parasito-proteomics studies have contributed to the discovery of candidate genes and new biochemical pathways potentially involved in parasitic manipulation. Future work should build upon this promising start. We suggest some additional considerations to move this work forwards. For instance, existing parasito-proteomics studies are missing: (i) the insoluble proteome linked to the manipulative process; (ii) the neuropeptidome response; and (iii) the host proteome response in a molecular weight (Mw) range of 20 kDa or less and a pH range 4 or less and 7 or greater. In addition, functional analysis in association with behavioural assays and interactome bioassays (see Box 3.2) will be necessary to confirm the involvement of the candidate proteins. Thus, a new integrative approach is necessary to bridge the gaps in our knowledge of how parasites manipulate their hosts (see Fig. 3.4).

Several new proteomics tools have been developed and can be used in the understanding and the deciphering of the manipulative process. For instance, SELDI-TOF can provide a complementary visualisation technique to two-dimensional (2D) electrophoresis. SELDI-TOF is more sensitive and requires smaller amounts of proteins than 2D electrophoresis (Bischoff and Luider, 2004; Issaq *et al.*, 2002; Seibert *et al.*, 2004). SELDI-TOF is most effective at profiling low Mw proteins (i.e., $<$20 kDa) and permits a rapid comparison of the host CNS proteome for many treatments by taking into consideration many physiochemical characteristics of proteins using SELDI protein chips with various chemical surfaces (hydrophobic, cationic, anionic, hydrophilic and metal ion preventing)

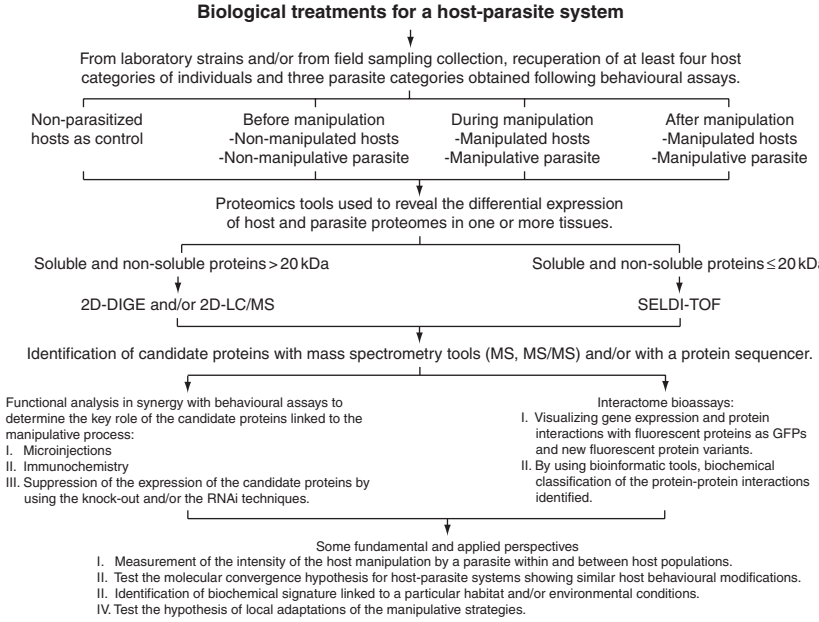

FIGURE 3.4 New integrative approach to study the proximate mechanisms in any host–parasite system. 2D-DIGE, two-dimensional-difference gel electrophoresis; 2D-LC/MS, 2 dimensional liquid chromatography/mass spectroscopy; GFP, Green Fluorescent Protein; MS, mass spectroscopy; RNAi, small fragments of double-stranded RNA; SELDI-TOF, surface enhanced laser desorption/ionization time-of-flight.

(Bischoff and Luider, 2004; Issaq *et al.*, 2002; Sanchez *et al.*, 2008; Seibert *et al.*, 2004). In the previous parasito-proteomics studies, no data were obtained about these key molecules (i.e., peptides and neuropeptides) influencing the physiological processes involved in the expression of host behaviour. For the proteins with a Mw greater than 20 kDa, the multi-dimensional liquid chromatography/mass spectrometry (LC/MS) offers a promising alternative and complementary approach to 2D electrophoresis for the analysis of complex protein mixtures. Multi-dimensional LC/MS has increased in popularity because this technique is relatively straightforward, the available software is convenient to use and once protein fractions are 'spotted' on matrix-assisted laser desorption/ionisation (MALDI) targets there are no time constraints on carrying out further analysis for the protein identification (Brand *et al.*, 2005; Greibrokk *et al.*, 2005). However, the 2D-difference gel electrophoresis (2D-DIGE) remains a very efficient option for the analysis of the differential expression of common proteins between different treatments.

3.2.2.3. Summary

The proximate mechanisms mediating changes in host behaviour are complex. This complexity probably exists because these mechanisms evolved from the mechanisms required for the survival of the parasite within the host (see also Combes, 2005). Given the fortuitous nature of evolution, it is not surprising that parasites influence host behaviour using multiple methods (see Fujiyuki *et al.*, 2005; Tomonaga, 2004). We will need a greater understanding of parasito-proteomics, immune-neural interactions (see Adamo, 2008; Dantzer *et al.*, 2008) and a more neuroethological approach to understand how parasites manipulate their host's behaviour fully. This necessitates an increase in our own 'crosstalk' with researchers investigating the proximate mechanisms of behaviour, such as neuroimmunologists and neurobiologists.

BOX 3.3 The three main types of manipulation

A. Manipulation *sensu stricto*

Host behavioural alteration may be regarded as a compelling illustration of the extended phenotype (Dawkins, 1982), that is, the expression of the parasite's genes in the host phenotype. The extended phenotype perspective thus postulates that in some host–parasite interactions the parasite genes are responsible for the aberrant behaviour. In this view, genes of the parasite are selected for their effect on host behaviour.

B. Exploitation of host compensatory responses

Host behavioural alteration may be regarded as a host response to parasite-induced fitness costs. Parasites may affect fitness-related traits in their hosts such as fecundity and survival in order to stimulate host compensatory responses because these responses can increase parasitic transmission. In this view, genes of the parasite are selected for their pathological effects that induce a host compensatory response. Since behavioural changes both mitigate the costs of infection for the host and meet the objectives of the parasite in terms of transmission, natural selection is likely to favour all the genes involved in this interaction.

C. Mafia like manipulation

Host behavioural alteration may be regarded as a forced collaboration. Parasites may select for collaborative behaviour in their hosts by imposing extra fitness costs in the absence of compliance. The parasite would be able to adopt a plastic strategy (i.e., facultative virulence) depending on the level of collaboration displayed by the host. In this view, genes of the parasite are selected for their ability to detect non-collaborative behaviours and their ability to produce retaliatory behaviour.

3.3. A CO-EVOLUTIONARY PERSPECTIVE

Parasitic manipulation often dramatically reduces host fitness. For this reason, the hypothesis of 'manipulation *sensu stricto*' is commonly seen as a game with evident winners (i.e., parasites) and losers (hosts) (Wellnitz, 2005) (Box 3.3(A)). The ability of parasites to manipulate host behaviour results from a long-term co-evolutionary interaction that probably leads to the mechanisms being complex (Section 3.3). Co-evolutionary dynamics implies that the host behavioural changes should thus be considered as an equilibrium state, a compromise resulting from an on-going arms race rather than a total parasite takeover (Poulin *et al.*, 1994; Wellnitz, 2005). From an evolutionary point of view, these considerations are relevant as they suggest that behavioural changes in infected hosts, even when they result in clear fitness benefits for the parasite, are not necessarily pure illustrations of the extended phenotype of the parasite. In a host–parasite system, natural selection is acting on the host genome as well. At present, very few studies on manipulative changes have explored the degree to which parasite-manipulated behaviours could be a compromise between the strategies of host and parasite. We present here two scenarios in which parasitic '*manipulation*' can enhance host fitness as well.

3.3.1. Exploiting host-compensatory responses

In this section, we propose that certain parasites could affect fitness-related traits in their hosts (e.g., fecundity, survival, growth, competitiveness, etc.) in order to stimulate host compensatory responses because these host responses enhance parasitic transmission (Lefèvre *et al.*, 2008) (Box 3.3B).

3.3.1.1. Compensatory responses in the living world

The phenotype of an organism results from both its genotype and the environment in which the genes are expressed. Phenotypic plasticity is the capacity of a genotype to express different phenotypes under different environmental conditions (Pigliucci and Preston, 2004). When faced with adverse environmental conditions, many organisms are able to alter some life history traits resulting in reduced fitness loss (Metcalfe and Monaghan, 2001). For instance, when facing potential resource limitations, plants possess a remarkable degree of developmental plasticity that enables them to balance their resource acquisition and maximise their fitness (Wise and Abrahamson, 2005). Similarly, it can be adaptive for parents in many animal species to avoid producing poor-quality

offspring when food is rare (e.g., by re-absorbing embryos or by reducing the production of offspring of the more vulnerable sex, see Uller *et al.*, 2007). Animals can also respond to adverse environmental conditions by using fecundity compensation (i.e., reproducing earlier in life and producing more offspring). For instance, in the presence of predatory fish, the cladoceran crustacean *Daphnia galeata* reproduce early and produce larger clutches of smaller offspring (Sakwinska, 2002).

Parasites influence the optimal strategies of their free-living hosts. Like other environmental factors, parasites have the potential to play an important role in the evolution of plastic compensatory responses. Selection will favour hosts that will react to parasite-induced fitness cost by adjusting their life history traits when they cannot resist infection by other means (e.g., immunity). Several theoretical and empirical studies back up this assumption by showing that infected hosts can adjust their reproductive effort or growth in such a way as to increase their fitness. For example, parasitised hosts react to a fitness loss due to infection via mechanisms such as an increased rate of egg laying (Adamo, 1999; Minchella and Loverde, 1981), enhanced courtship behaviour (McCurdy *et al.*, 2000; Polak and Starmer, 1998), higher offspring number and/or size (e.g., Kristan, 2004; Sorci and Clobert, 1995) and/or stronger parental effort (Christe *et al.*, 1996, Hurthrez-Boussès *et al.*, 1998; Tripet and Richner, 1997). High risk of infection can also select for early-onset sexual maturity in the entire population (Agnew *et al.*, 1999; Fredensborg and Poulin, 2006; Lafferty, 1993). In other cases, hosts compensate by diminishing their reproductive effort, presumably to enhance survival, which could in return increase the probability of outliving or sequestering the parasite (Forbes, 1993; Hurd, 2001; Sorensen and Minchella, 2001). Therefore, compensatory changes in behaviour may be a widespread strategy among organisms facing adverse conditions such as parasitism.

In some cases, parasites can exploit host compensatory responses that have been selected in other ecological contexts by mimicking the causes that induce them. In other cases, parasites themselves can be the triggers of the compensatory response because of their significant effects on host fitness.

3.3.1.2. Empirical support

To our knowledge, theoretical and/or experimental studies specifically designed to test this scenario have never been carried out. However, in the literature there are several examples of parasite-induced phenotypic changes that have been interpreted either as cases of adaptive host responses or manipulation *sensu stricto* but these same cases could, in fact, illustrate the exploitation of host compensatory responses. Below, we present some of these examples.

3.3.1.2.1. Foraging activity

(a) *Predation risk*. An increased predation risk is a change that can potentially be of interest for trophically transmitted parasites since, by definition, this type of parasite requires a predation event to complete its life cycle. Parasitised hosts often have increased energy requirements and forage more to compensate for the negative effect of infection. However, until now the subsequent increased predation risk has been traditionally viewed as a by-product of the infection that is coincidentally beneficial for the parasite (but see Thomas *et al*., 2005). Parasites live at the expense of their hosts, and consequently there are many reasons, other than transmission, for parasites to divert energy away from the host (growth, maturation of gonads). We agree with this parsimonious way of thinking (Box 3.1) but we feel that in the present evolutionary context, parsimony can be viewed differently. Host exploitation by parasites can potentially affect a broad range of fitness-related traits in hosts such as survival, fecundity or sexual attractiveness. These phenomena are expected to favour the evolution of compensatory responses in the host, such as an increased foraging or reproductive activity. For instance, three-spined sticklebacks (*Gasterosteus aculeatus*) infected by the cestode *Schistocephalus solidus* exhibit marked differences in their anti-predator, foraging and shoaling behaviour compared with uninfected conspecifics (Barber and Huntingford, 1995; Godin and Sproul, 1988; Ness and Foster, 1999). The increased nutritional demand of parasitised fish (Pascoe and Mattey, 1977) may stimulate foraging behaviour that exposes them to greater predation risk than uninfected counterparts (Godin and Sproul, 1988; Milinski, 1985). This example has been interpreted as an illustration of the 'side-effect' hypothesis according to which these changes result from pathological effects of infection that are coincidentally beneficial for the parasite (Box 3.1; Poulin, 1995). However, the behavioural changes observed in sticklebacks infected by *S. solidus* are consistent with the view that the fish benefits by obtaining more food to compensate for the resources taken by the cestodes. Thus the host will gain, at least until predated, and by that time it could have reproduced. The parasite clearly gains by making the host more vulnerable to predation. However, one cannot exclude active manipulation *sensu stricto* of host neuroendocrine systems by the parasite, for instance by the release of a neuroactive substance (Overli *et al*., 2001). What we wish to emphasise here is that competing ideas need consideration when searching for proximate mechanisms of manipulation.

(b) *Qualitative change*. It has been frequently reported that parasitised organisms change their foraging behaviour (Moore, 2002). If foraging leads to increased uptake of resources that can help fight the infection

it is often seen as a case of self-medication (Hart, 1994). When infected with the tachinid parasitoid *Thelaira americana*, the caterpillar host *Platyprepia virginalis*, changes its feeding preference from lupine to hemlock (Karban and English-Loeb, 1997). This change apparently reduces the costs of the infection for the host because infected caterpillars feeding on hemlock survived the emergence of the parasite and even metamorphosed into sexually mature adults without losing fecundity (English-Loeb *et al.*, 1990, 1993). This response also seems to be beneficial to the parasite. The pupal mass of flies (a good correlate of fecundity) emerging from caterpillars reared on hemlock was indeed greater than that emerging from lupine-fed caterpillars (Karban and English-Loeb, 1997). In this example both host and parasite interests are aligned (Dawkins, 1990).

(c) *Biting behaviour in haematophagous insects*. When haematophageous insects feed on their hosts, they are liable to transmit many pathogens. Vector-borne parasites manipulate several phenotypic traits of their vertebrate hosts and vectors in ways that favour parasite transmission (Hurd, 2003; Lefèvre and Thomas, 2008; Molyneux and Jefferies, 1986; Moore, 2002; Section 3.3). For instance, infected-insect vectors seem to develop an increased probing and feeding rate (e.g., tsetse flies infected with African trypanosomes, Jenni *et al.*, 1980; Roberts, 1981; bugs infected with *Trypanosoma* spp., Anez and East, 1984; Botto-Mahan *et al.*, 2006; Garcia *et al.*, 1994; sandflies infected with *Leishmania* spp., Beach *et al.*, 1985; Killick-Kendrick *et al.*, 1977; Rogers and Bates, 2007; fleas infected with plague bacterium, Bacot and Martin, 1914; Gage and Kosoy, 2005; mosquitoes infected with *Plasmodium* spp., Koella *et al.*, 1998, 2002; Rossignol *et al.*, 1986; Wekesa *et al.*, 1992; and viruses, Grimstad *et al.*, 1980; Platt *et al.*, 1997). Increased biting is usually associated with mechanical interference, that is, the vector's ability to engorge fully is impaired and therefore this induces them to bite vertebrate hosts several times (Hinnebusch *et al.*, 1998; Molyneux and Jenni, 1981; Rogers and Bates, 2007). This would appear to be manipulation *sensu stricto*. However, Rossignol *et al.* (1986) demonstrated reduced fertility in *Aedes aegypti* parasitised with *Plasmodium gallinaceum*. When infected mosquitoes were free to bite more, they recovered a normal level of fecundity (i.e., equal to uninfected conspecifics). In this view, the increased biting rate of *A. aegypti* may represent a host compensatory response to parasite-induced fecundity reduction.

3.3.1.2.2. Sexual behaviour Longevity and reproduction are crucial fitness determinants of most organisms (Clutton-Brock, 1988). A trade-off between these two key life-history traits is expected so that reductions in longevity leads to increased reproductive effort (Polak and

Starmer, 1998). Parasites often reduce the survival of their host, and infected hosts are expected to respond by increasing their reproductive effort. Parasites with direct transmission could benefit from decreasing the reproductive output of their host. Decreased offspring production should promote a compensatory increase in sexual behaviour, and hence parasite transmission. The sexually transmitted ectoparasite, *Chrysomelobia labidomera*, reduces the survival of its leaf beetle host (*Labidomera clivicollis).* In response, infected males exhibit increased sexual behaviour before dying (Abbot and Dill, 2001). The host compensation hypothesis predicts a positive relationship between parasite load and reproductive effort (Forbes, 1993; Polak and Starmer, 1998). As expected, the study by Abbot and Dill (2001) showed a positive relationship between male parasite load, the frequency of sexual contact and duration of copulation. This behavioural modification clearly benefits the sexually transmitted parasite since enhanced inter- and intra-sexual contact (i.e., copulation and competition) provide more opportunities for transmission (Abott and Dill, 2001; Drummand *et al*., 1989).

In the same vein, it has been reported that females of the amphipod *Corophium volutator* compensate for the negative effect of the trophically transmitted trematode *Gynaecotyla adunca* on survival by increasing their reproductive activity (McCurdy *et al*., 2000, 2001). Males appeared to compensate for parasitism by being more likely to mate, and perhaps by increasing ejaculate size. In amphipods mating occurs only during a narrow part of the female's moult cycle. Since moulting is asynchronous, the operational sex ratio is strongly male biased, and males compete for access to larger, more fecund females. In response, pre-copulatory mate guarding has evolved in amphipods. Interestingly, such behaviour is known to increase the predation risk because pairs are more conspicuous, less manoeuvrable and more profitable as prey than single individuals (Cothran, 2004; Ward, 1986). Thus one can hypothesise that parasites trigger host fecundity compensation because host mating increases the chance of being preyed upon by the definitive hosts. This example, however, must be considered carefully because the increased sexual activity of parasitised gammarids may occur before the trematode is infective to vertebrate predators.

3.3.1.2.3. Inclusive fitness In the Hawaiian Islands, corals from the genus *Porites* are susceptible to infection by the digenetic trematode *Podocotyloides stenometra* (Aeby, 1991, 1992). This parasite has a complex life cycle involving a molluscan as first intermediate host, *Porites* as the second intermediate host, and coral-feeding fish as the final host. *Porites* infected with this trematode display pink swollen nodules. Given that these parasitised polyps represent a burden for the coral (reduced growth), the coral would benefit from eliminating and replacing them, for example, by

offering them to predators. As a matter of fact, the Butterfly fish (definitive host) do prefer the parasitised polyps and hence contribute to the regeneration of a healthy polyp (Aeby, 1992). Whereas the higher susceptibility to fish of infected polyps seems to be a case of host manipulation *sensu stricto* by a parasite, it also agrees with the idea that the parasite relies on host compensatory responses for its transmission.

3.3.1.2.4. Gigantism Many parasite species can reduce host fecundity, either partially or via full castration, by channelling energy away from host reproduction toward their own growth (Poulin, 2007). This fecundity reduction often results in host gigantism, especially in molluscs serving as first intermediate hosts of larval trematodes (Minchella, 1985). This phenomenon is consistent with the idea that phenotypic changes following infection can be considered as co-evolved traits. As size and fecundity are positively correlated in snails, the parasitised hosts can benefit from investing energy in growth, with fecundity compensation occurring later, after the death of the parasite. However, the parasite remains the first beneficiary of such a compensatory strategy since the larger size of the host allows the parasite to increase the biomass of the sporocyst and thus produce thousands of infective larvae.

3.3.1.3. Future directions

Most studies on parasitic manipulation assume that host phenotypic changes that benefit the parasites are compelling illustrations of the extended phenotype (*sensu* Dawkins, 1982; but see Ponton *et al*., 2006b), that is, the expression of the parasite's genes in the host phenotype. The perspective presented above attempts to balance this view. We suggest that changes in host behaviour, even those that benefit the parasite, can be due to compromises between host and parasite strategies (i.e., a shared phenotype).

To our knowledge, it is novel to consider that parasites could achieve transmission by triggering host compensatory responses, when the latter fit (totally or in part) with the transmission route. Is this strategy common? Further studies are clearly needed at the moment to answer this question, but it may be a widespread strategy. This type of host manipulation seems parsimonious for several reasons when compared with the hypothesis of manipulation *sensu stricto*, in which the parasite must maintain a certain degree of manipulative effort with putative fitness costs. Indeed, if among the arsenal of compensatory responses displayed by the host, some are beneficial for transmission, selection is likely to favour parasites that exploit these responses, not only because this meets their objectives, but also because this requires no manipulative effort: the host is doing the job. Another good reason to believe that exploiting host compensatory responses is a likely scenario from an evolutionary

perspective comes from the fact that it is also advantageous for the host: once infected, it is better for the host to behave in a way that alleviates the costs of infection, even when this also ultimately benefits the parasite (aligned desiderata, Dawkins, 1990). Under these conditions, resistance is less likely to evolve than when there is no compensation for the host.

Based on these considerations, we could predict that manipulation *sensu stricto* will exist most often in systems in which there are no host compensatory mechanisms that would result in increased parasite transmission. As a possible example of such a situation, we suggest the case of the well-known example involving the small liver fluke (*Dicrocoelium dendriticum*). It is indeed difficult to imagine what kind of compensatory responses could make the ant climb to the tip of a grass blade.

Besides the relevance of considering host compensatory responses in the context of transmission strategies, we believe that it could also be a promising approach for the study of many other aspects of host–parasite relationships.

Natural selection should favour parasites that impose specific costs on their host (with a precise schedule adjusted by selection) each time there is a host compensatory response that is beneficial for them. In our opinion, these ideas are very promising for the understanding of the ultimate basis of parasite pathogenicity and virulence (Lefèvre *et al.*, 2008).

3.2.2. Facultative virulence

The mafia-like strategy of manipulation is probably the most extreme scenario demonstrating the interactive nature of the relationship between parasites and hosts (Zahavi, 1979). This strategy suggests that parasites may select for collaborative behaviour in their hosts by imposing extra fitness costs in the absence of compliance. In this scenario the parasite would be also able to adopt a plastic strategy (i.e., facultative virulence) commensurate to the rate of collaboration displayed by the host. In response to a host's opposition to manipulation, a parasite could increase virulence because the host does not behave as expected. Therefore, non-collaborative behaviours are a more expensive option for the host than collaborative ones. This 'mafia-like strategy' can, in theory, force the host to accept behaving in ways that benefit the parasite (Box 3.3(C)). Here, we discuss and review possible evidence around this idea.

3.2.2.1. Host–parasite interactions and state-dependent models

Both the host and the parasite must be able to adjust their life history decisions in a state-dependent manner for the mafia strategy of manipulation to evolve. Numerous lines of evidence suggest that free-living organisms are able to recognise environmental cues, including parasitic infection, and to adjust their life history traits accordingly (Section 3.3.1.1).

There are recent suggestions that parasites are also able to perceive a large set of environmental variables and respond to these in a state-dependent manner (thereby maximising their lifetime reproductive success) (Lewis *et al*., 2002; Thomas *et al*., 2002b). Parasites are, for instance, expected to recognise many physiological and biochemical conditions of their internal host environments that are of selective importance (age and sex of the host, presence/absence of other parasites). There are also good reasons to believe that parasites are able to perceive cues concerning the external environment of their hosts. For example, parasites can respond to host population density, the presence of predators, or the presence of sexual partners or competitors (see Thomas *et al*., 2002b). Poulin (2003) provided empirical evidence that the environmental perception of parasites can be much more sophisticated than traditionally thought. The trematode *Coitocaecum parvum* from New Zealand is able to accelerate its development and reach precocious maturity in its crustacean intermediate host in the absence of chemical cues emanating from its fish definitive host. Juvenile trematodes can also mature precociously when the mortality rate of their intermediate hosts is increased (Poulin, 2003). These results show that growth decisions and developmental strategies in this parasite are plastic, and conditional upon the opportunities for transmission. More generally, these results suggest that parasites can exploit several sources of information both internal and external to the host.

3.3.2.2. Mafia strategy of manipulation

By imposing extra fitness costs in absence of compliance, parasites have the potential to select for collaborative behaviour in their hosts. Of course, these collaborative behaviours do not result from conscious choices. Over time, selection is expected to produce shifts in the behaviour of infected individuals if such a shift increases their chance of survival and reproduction. In some systems, hosts that alter their behaviour in such a way that benefits the parasite may have better survival and more offspring than infected hosts that do not.

3.3.2.3. Empirical support

The cuckoo is the best exemplar of the mafia hypothesis. Zahavi (1979) hypothesised that cuckoos force their hosts to tolerate non-self eggs by making the consequences of rejection more damaging than acceptance. Soler *et al.* (1995) studied the relationship between the great spotted cuckoo (*Clamator glandarius*) and its magpie host (*Pica pica*). In this host–parasite system, the host can raise at least part of its own young along with those of the cuckoo. Soler *et al.* (1995) showed that ejector magpies suffered from considerably higher nest predation levels by cuckoos than did accepters. The interpretation being that the cuckoo retaliates and punishes non-compliant hosts. As a result, the frequency of 'accepting

genes' is more likely to increase in the host population than 'rejecting genes' (Soler *et al*., 1999). In an area with a high density of cuckoos, Soler *et al.* (1998) showed that magpies that rejected cuckoo eggs from their first clutch were more likely to be parasitised by cuckoos during their second clutch than magpies that accepted the cuckoo eggs during the first clutch.

Pagel *et al*. (1998) modelled the evolution of retaliation by brood parasites. Retaliation evolves even when hosts rear only the parasite's young (its own offspring having been ejected by the parasite, which is the case when nests are parasitised by *Cuculus canorus*). This is possible if, during the breeding season, non-ejectors enjoy lower rates of parasitism in later clutches compared to ejectors, making non-ejectors able to rear a clutch of their own following the rearing of a cuckoo nestling, while ejectors are likely to be re-parasitised. Pagel *et al.* (1998) stressed that, for this scenario to function, it implies that brood parasites have a good memory for the location and status of nests in their territory.

Recently, Hoover and Robinson (2007) provided experimental evidence for the mafia strategy in the brood parasite, the brown-headed cowbird (*Molothrus ater*). In manipulating ejection of cowbird eggs and cowbird access to nests of their warbler host, they showed that 56 % of ejector-nests compared with only 6% of accepter-nests were destroyed by cowbirds (Hoover and Robinson, 2007). This mafia behaviour selects for collaborative hosts not only in evolutionary time by decreasing the proportion of hosts that bear rejector genes, but also within the lifetime of an individual host through a learning process. Learning probably occurs in parasitic systems in which individual host females are likely to be parasitised repeatedly within or across breeding seasons. In addition, the authors also showed that collaborative behaviours benefit the hosts as well, since warblers produced significantly more offspring by complying with the parasite.

3.3.2.4. Future directions

Examples of mafia strategy of manipulation remain scarce at the moment, but this is likely to reflect a lack of appropriate studies. We encourage researchers to imagine experiments that place infected hosts in a situation of 'disobedience' as regard to what they should do to benefit the parasite, and to study the fitness consequences of such non-compliance. It would also be necessary to determine whether pre-adaptations (physical location of the parasite with respect to the host, number and kind of hosts involved in the life cycle and phylogenetic constraints) exist for behavioural changes. In addition, do these factors matter more in cases of manipulation *sensu stricto*, than in one of the 'interactive' strategies presented above? Knowing that manipulative costs should, in theory, be lower for parasites when the host has some fitness compensation in performing the altered behaviour, we might even expect that the transition from 'pure'

manipulation to 'interactive' strategies of manipulation is likely to be a scenario favoured by selection. Finally, this co-evolutionary perspective suggests that host behavioural changes can benefit the host even if they also benefit the parasite.

3.4. THE (RIVER) BLIND WATCHMAKER

In what is the most well-known argument from design, the Reverend William Paley (1802) said that just as we conclude that a watch we find lying on the ground must have had a creator, then so too must other complex things such as animals have had a creator. In Paley's case, the creator was divine but since the publication on the *Origin of Species* (Darwin, 1859), we now accept the theory of natural selection as a more satisfying explanation. In defending Darwin's theory Richard Dawkins (1986) said of natural selection that ''*It has no mind and no mind's eye. It does not plan for the future. It has no vision, no foresight, no sight at all. If it can be said to play the role of watchmaker in nature, it is the blind watchmaker*''.

How well do we understand the interactions between parasites and their hosts? In many cases we have an enviable level of understanding of these interactions (e.g., our understanding of the antigenic variation of the protein coat of malaria, Schmid-Hempel, 2008). However our understanding is far from complete, even though parasites have the reputation of being simple organisms. (Admittedly it is non-parasitologists putting forwards this view.) Parasites are generally reduced in morphology. Also, their genomes are often reduced compared to free-living relatives (Keeling and Slamovits, 2005). The effects that parasites have on their host are likewise viewed as crude. River blindness, caused by the nematode, *Onchocerca volvulus*, is a case in point. Adult worms produce thousands of microfilaria each day and these cause a range of symptoms that often occur after these immature stages die in the human host without ever being transferred to the fly vector. The most infamous effect of these larvae is the scarring of the eye leading to blindness. River blindness causes great morbidity and mortality in hosts and reinforces the view that parasites appear to take a sledgehammer approach to the host (Section 3.3.1).

By contrast the specific changes in host behaviour that are observed in some systems can be viewed as the parasite extending its phenotype and taking control of the actions of the host. In our review we have sought to temper that view by saying that in many cases parasites appear to induce multiple and widely disseminated changes in their hosts' CNS as opposed to targeted attacks on specific neural circuits. Moreover, the behavioural change is not always the sole property of the parasite: the reaction of the host may also be important.

3.5. CONCLUDING REMARKS

When presented with a parasite causing an elaborate and often times bizarre behavioural change in its host, an obvious question that arises is how? Yet, by any admission, the field has been overly focused on why (i.e., explaining the behaviour in an adaptationist framework where the fitness benefit is ascribed to the parasite, host or neither (Box 3.1)). In this review we considered the evidence of how parasites induce changes. Overall, the evidence is slight and even the best-studied examples require further data before behavioural changes can be considered parasite manipulation in its most strict sense. Recognising this short fall we have further tempered the manipulation *sensu stricto* view by pointing to other factors such as host immune responses, compensatory responses and facultative virulence. Our goal has been to present the current evidence for parasitic manipulation of host behaviour. To move forwards we require less debate and more evidence. How might this be achieved?

Researchers interested in behavioural manipulation need a fuller discourse with colleagues who understand how physiology, neuroanatomy and omics contribute to behavioural trait expression. This is requisite to avoid situations where the evidence of some aberration (e.g., hormone titres, smaller brain regions or distinctive proteomes) is taken as evidence of adaptive manipulation. It is possible and probable that other scenarios in uninfected hosts (e.g., stress, senescence) lead to similar signatures. In addition to greater collaboration, the field might benefit from focusing on some systems that could be developed into models of host–parasite manipulation events. Clearly, some of those reviewed above would be good contenders. In a related vein, genomic approaches will herald a new era in understanding how parasites control behaviour. A goal of the field should be a full understanding of the proximate mechanisms of how a parasite affects host behaviour. It is our hope that one day a collaborative and multi-disciplinary research approach will be able to peel back a particularly compelling example of an extended phenotype to shows its physiological, neurological and ultimately its genetic basis. Then we will know how parasites manipulate a host.

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CHAPTER 4

Evolutionary Drivers of Parasite-Induced Changes in Insect Life-History Traits: From Theory to Underlying Mechanisms

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Abstract

Many hosts are able to tolerate infection by altering life-history traits that are traded-off one against another. Here the reproductive fitness of insect hosts and vectors is reviewed in the context of theories concerning evolutionary mechanisms driving such alterations. These include the concepts that changes in host reproductive fitness are by-products of infection, parasite manipulations, host adaptations, mafia-like strategies or host compensatory responses. Two models are examined in depth, a tapeworm/beetle association, *Hymenolepis diminuta/Tenebrio molitor* and malaria infections in anopheline mosquitoes. Parasite-induced impairment of vitellogenesis ultimately leads to a decrease in female reproductive success in both cases, though by different means. Evidence is put forwards for both a manipulator molecule of parasite origin and for host-initiated regulation. These models are backed by other examples in which mechanisms underlying fecundity reduction or fecundity compensation are explored. It is concluded that evolutionary theories must be supported by empirical evidence gained from studying molecular, biochemical and physiological mechanisms underlying changes in host life-history traits, ideally using organisms that have evolved together and that are in their natural environment.

4.1. PARASITES AND HOST LIFE-HISTORY TRAITS

Hosts can reduce the detrimental effects of parasitism by erecting barriers to initial invasion or by mounting a defensive attack to clear or suppress an infection. All such measures are likely to incur costs in the host (Sheldon and Verhulst, 1996) and result in the evolution of countermeasures by the parasite. An alternative way of dealing with the threat of infection is tolerance (Corby-Harris *et al.*, 2007). This is likely to involve a different, or additional, strategy; namely for the host's life-history traits to alter in response to infection (Agnew *et al.*, 2000).

The life-history traits of an organism, such as growth, life-time reproduction and longevity, interact at a physiological level. As they are constrained by the organism's total resources, one trait will be traded-off against another. For example, life-history theory assumes that the costs of

current reproduction will be traded-off against future survival and reproductive effort. Different allocations will be made in different situations and will result in optimisation of fitness (Stearns, 1992).

It has long been recognised that parasites and pathogens can act as agents in the evolution of host life-history strategies and in the maintenance of plasticity and diversity of these traits within populations. As well as robbing nutrients from its host, the presence of a parasite may result in changes in such things as growth rate, mating success, fecundity or life span. Although these alterations to life-history traits are likely to be costly to the host, the infected organism may ultimately be fitter than if they had not occurred (Hurd, 1998). In particular, diversion of some resources away from reproduction, at a time when parasites are invading or growing rapidly, may avert early host death due to parasite virulence, thereby increasing the potential life span of the host–parasite complex. In doing so the parasite will gain extra time in which to mature and undergo transmission and the host may gain extra time during which additional mating may occur or eggs be produced. Fecundity may thus be traded off against life span (possibly by using the additional resources that are released to mount an attack on the parasite) such that decreased fecundity increases life span. If a longer life span equals more time to reproduce, thereby outweighing the original reproductive loss, then host fitness is enhanced.

This article reviews current thinking concerning the effect of parasitic infection on life-history traits, focusing upon reproductive effort. Insects have proved to be useful models with which to investigate life-history traits and most of the examples used herein are drawn from parasite/insect associations.

4.2. VARIOUS CHANGES IN REPRODUCTIVE TRAITS SEEN IN INFECTED INSECTS

Insects belong to a large and diverse taxa and are afflicted by a plethora of microbe, parasite and parasitoid infections (Hurd, 1993). Insect eggs are generally well provisioned with yolk protein and other ingredients essential for the development of the embryo, thus egg production is very costly to the female insect. Other costly aspects of reproduction include male courtship and mating behaviour. The effort that insects invest in reproduction is altered by the physiological and environmental conditions pertaining at the time. For example, fruit flies abort the development of ovarian follicles and resorb maturing oocytes when they encounter adverse conditions such as lack of food or sex peptide from the male (Rauschenbach *et al.*, 2004; Soller *et al.*, 1999). There are numerous examples of the effect of parasites on insect reproductive effort, many of which have been described in past reviews that will be cited in this text.

4.2.1. Fecundity reduction

In insects, oogenesis and oviposition are controlled by a complex hierarchy of endocrine and neural signals that respond to the environment and the physiological status of the female. In many cases, these regulatory mechanisms respond to the presence of parasites by down-regulating reproductive effort, either temporarily or permanently. This reproductive curtailment takes a variety of forms, including juvenilisation, destruction of reproductive tissue, changes in sexual behaviour, maturation of fewer eggs or a decrease in egg fertility. Examples of parasites that induce fecundity reduction are widespread and can be drawn from microsporidia, protozoa, nematodes, cestodes, mites and endoparasitic insects, as reviewed by Hurd (1993). Some examples will be described below and two discussed in detail later in this article.

4.2.1.1. Loss of female reproductive fitness

Beetles of the family Tenebrionidae exhibit a marked long-term depression in population size when infected with the metacestodes of the rat tapeworm, *Hymenolepis diminuta* (reviewed by Hurd, 1990). There is a retardation of the development of oocytes in the ovaries of infected *Tenebrio molitor* and some of the follicles fail to develop and are resorbed (see Hurd and Webb, 1997 for a review and Warr *et al.*, 2006b). Similarly, mosquitoes infected with various species of the malaria parasite, *Plasmodium* spp., produce fewer eggs (reviewed by Hurd, 2003 and see Gray and Bradley, 2006; Marrelli *et al.*, 2007). In some mosquito-malaria associations it has been shown that, here too, fecundity reduction is caused by a retardation of oocyte maturation and the resorption of ovarian follicles (Hopwood *et al.*, 2001). In *Anopheles gambiae*, resorption occurs as a result of infection-induced apoptosis of follicular epithelial cells (Ahmed *et al.*, 2002). Several other vector–parasite associations have been shown to result in fecundity reduction, including filarial worm-infected mosquitoes (Christensen, 1981; Javadian and Macdonald, 1974), fungal-infected mosquitoes (Scholte *et al.*, 2005), *Leishmania*-infected sandflies, (el Sawaf *et al.*, 1994) and *Onchocerca*-infected blackflies (Ham and Banya, 1984; Renshaw and Hurd, 1994). Finally, mermithid nematodes reduce the fecundity of a variety of insects and cause a complete inhibition of vitellogenesis, resorption of ovarian follicles and permanent cessation of egg production in locusts (reviewed by Hurd, 1990).

4.2.1.2. Loss of male reproductive success

The adult phonotactic fly, *Therobia leonidei* is acoustically orientated towards singing male bush crickets, including *Poecilimon mariannae*. Parasitism substantially reduces male potential life-time reproductive success, not only by reducing survival (males die when the parasitoid larva

emerges) but also by reducing calling effectiveness, by decreasing the attractiveness of the call to females and by reducing the size of the spermatophylaces. No evidence of reproductive compensation occurs, despite the inevitable death of the host (Lehmann *et al.*, 2006). Parasitoid-induced alteration of sexual singing is thus sending an honest signal of male infection status to females and enables infection-mediated sexual selection. There is evidence that this change in sexual signalling in male crickets is mediated by an immune response. Immune induction via injection of the bacterial wall component lipopolysaccharide (LPS) caused a long-term change in male calling that was independent of nutritional status and was accompanied by a reduction in life span in a field cricket (Jacot *et al.*, 2004) and provision of paternal resources in a ground cricket (Fedorka *et al.*, 2007).

4.2.2. Fecundity compensation

Rather than immediately inducing a curtailment of reproductive effort, some parasites have been shown to enhance the early reproductive success of their hosts. This can take the form of a higher output of offspring early in life or an earlier age at which reproduction first occurs. Enhanced early reproductive success is a phenomenon known as fecundity compensation. Hochberg and co-authors (1992) developed a mathematical model to predict the influence of a parasite population on host survival and time to begin reproduction. They concluded that parasite virulence and pathogenicity inversely affects host pre-reproductive life span but that this may be modulated by the evolution of defences against parasites. Adult-insect life span is generally short and reproductive maturity occurs soon after occlusion, thus there is little opportunity to hasten the onset of reproduction in the face of infection and examples of this are rare among insects. However, fecundity compensation (in some form) has been shown to occur in a few infected insects.

4.2.2.1. Early oviposition

Increased oviposition of stored eggs following bacterial infection has been observed in the crickets *Gryllus texensis* and *Acheta domestica* (Adamo, 1999; Shoemaker *et al.*, 2006). This is clearly adaptive behaviour on the part of the female, which is unlikely to survive the infection. Putative involvement of some, but not all, aspects of the cricket immune system has been suggested here as increased oviposition was also elicited by injection of LPS into *A. domestica*. Early oviposition does not, however, occur when an encapsulation response is induced by injected Sephadex beads or the presence of larvae of the parasitoid fly, *Ormia ochracea* (Adamo, 1999). Clearly, this form of fecundity compensation in orthopterans may provide a useful

model with which to study links between the immune and reproductive systems as discussed in Section 4.4.3.1.

The darkling beetle, *T. molitor*, was also shown to initially oviposit more eggs when infected with metacestodes of the tapeworm *H. diminuta*. However, as eggs are usually stored before oviposition, it is likely that those laid during the first few days of infection had already developed before the beetle was infected, as with the bacteria-infected crickets, and this is not a case of more resources being devoted to egg production at the onset of infection. Fertility was severely reduced over a longer time span and several aspects of beetle reproductive physiology were disrupted by infection as seen in Sections 4.4.1.1 and 4.4.1.2.

4.2.2.2. Nuptial gifts

H. diminuta infect male as well as female beetles. If males are infected on the second day following occlusion, the presence of maturing metacestodes induces the bean-shaped accessory glands to undergo an extended period of growth. By day eight post-infection they are 30–40% larger, contain more protein and produce spermatophores with significantly more protein and greater trehalase activity than glands from uninfected beetles of the same age (Carver and Hurd, 1998; Carver *et al.*, 1999). Uninfected females that mate with infected male beetles produce significantly more offspring than those mating with uninfected males and this increase is related to the intensity of infection in the males (Hurd and Ardin, 2003). It has been suggested that infected males are passing greater nuptial gifts to females, although, whether these include sex peptides is unknown (Hurd and Ardin, 2003). No increase in spermathecae trehalase activity or whole body juvenile hormone titre was detected in these females (Carver *et al.*, 1999; Cole *et al.*, 2003) and the mode of action of the putative nuptial gifts is not understood. Far from reducing the life span of infected males, *H. diminuta* increases the median survival by 4 days (Hurd *et al.*, 2001). Thus, in this case, enhanced reproductive potential is not a compensation for loss of later potential. However, it may counterbalance depressed production of, and response to, sex pheromones (Hurd and Parry, 1991).

4.2.2.3. Courtship behaviour

Although female *Drosophila nigrospiracula* experimentally infested with the mite *Macrocheles subbadius* suffer from reduced reproductive output due to early death (Polak, 1996), infested males are able to compensate for this loss by a change in courtship behaviour. Mating speed is significantly enhanced and copulation success is inversely related to longevity; the more heavily infected a male is, the more resources are devoted to reproduction and the shorter his life span (Polak, 1996; Polak and Starmer, 1998).

4.2.2.4. Early maturation

Acceleration in maturation, and hence early reproduction, is seen in *Culex pipiens* mosquitoes infected with microsporidian species. Rapid larval growth enhances the chances of the individual pupating before becoming overwhelmed by microsporidian spores; but this is at the expense of later reproductive success as these pupae give rise to smaller, less fecund females (discussed by Agnew *et al.*, 2000).

4.3. POTENTIAL DRIVERS OF CHANGE IN HOST REPRODUCTIVE SUCCESS

The examples given above illustrate how common and how varied phenotypic changes in reproductive strategies exhibited by parasitised insects can be. The origin of the drive for changes in host life-history trait has been much debated. Most of this debate has revolved around changes in behavioural traits that may be perceived to enhance the chances that parasite transmission occurs (see for example Thomas *et al.*, 2005). However, before a parasite can undergo successful transmission to the next host in its life cycle, a period of parasite growth, maturation and/or reproduction usually occurs. During this pre-patent period, the host must not only survive the infection but also provide all the resources that the parasite needs, or transmission will not occur. Changes in host phenotype that maximise conditions for the parasite during this period will thus ultimately enhance transmission prospects. Hypotheses concerning drivers for behavioural manipulation could thus equally be applied to changes in host reproductive success.

4.3.1. Pathological by-products of infection

Reproductive disturbances could be secondary outcomes of infection and of no adaptive value to either parasite or host. For example, they could be a direct consequence of tissue damage, as in the case of crickets infected with a tachinid parasitoid (Adamo *et al.*, 1995). However, incidences of this have rarely been recorded for insect host, possibly because, with the exception of maternally transmitted micro-parasites (that are outside the scope of this review), few parasites invade or destroy insect reproductive tissues.

Parasitic infections could cause a direct reduction in the supply of nutrients required to fuel reproductive behaviour or synthesise gametes. This would happen if host feeding is impaired or parasite needs exhaust stored reserves and cause a situation of host starvation or depletion of specific nutrients. Few studies have looked at the direct effect of parasitic infection on insect metabolites and linked this with fecundity reduction or

compensation. Gray and Bradley (2006) were unable to attribute fecundity loss in malaria-infected *Aedes aegypti* to reduced carbohydrate or lipid content. However, Ferdig *et al.* (1993) linked L-tyrosine deficits with reduced egg production in the mosquito/filarial worm association *Armigeres subalbatus*/*Brugia malayi*. This was proposed to be caused by an indirect effect of an amino acid requirement by the melanisation defence response mounted against the worm, rather than direct food robbery by the parasite.

Thomas and colleagues (2005) have serious reservations concerning the assignment of changes in host behavioural traits to the category of by-products of infection and their arguments can equally be applied to reproductive disturbances. With the exception of parasites that destroy gonad tissue, evidence that traits such as fecundity reduction or compensation have been altered as an accidental consequence of pathology caused by the parasite is rare. Moreover, the common occurrence of these responses to infection suggests they are unlikely to be accidental (Poulin, 1995).

4.3.2. Adaptive changes that may be driven by the parasite

Although alterations in host reproductive strategies, and in particular fecundity reduction, could be simple by-products of infection, most examples are more likely to result from the selection of adaptive strategies on the part of the host or of the parasite. Current thinking on the evolution of host responses to infection has been much influenced by Dawkins' concept of the extended phenotype (Dawkins, 1982, 1990). The idea that the product of a parasite gene could alter the host phenotype has been particularly explored in the context of changes in host behaviour, and many extraordinary and fascinating examples have been described (Moore's text provides a comprehensive review; Moore, 2002). However, it is only in the last two decades that these examples have been examined critically from an evolutionary standpoint (Poulin, 1994, 2000; Thomas *et al.*, 2005). The extended phenotype theory implies that a manipulator molecule of parasite origin is responsible for these changes and that the host is being manipulated directly by the parasite. Many of the criteria set by Poulin (1995) to define parasite manipulation of host behaviour would equally apply to parasite-induced changes in insect reproductive success (Hurd, 2005). It has arisen in several parasite/insect lineages, the underlying processes appear to be complex and one could argue that, in most cases, the parasite will gain if fewer host resources are devoted to reproductive effort. However, manipulation by the parasite implies the production of manipulator molecules that directly affect host reproduction and evidence for the existence of these is, as yet, scarce (but see Section 4.4.1.1).

4.3.3. Adaptive changes driven by the host

Many authors have assumed that changes in life-history traits in response to parasitic infection are adaptive mechanisms that are host directed and minimise the impact of infection. Forbes (1993) proposed that hosts would make changes in the degree and timing of reproductive effort depending upon the life-history pattern of the infecting parasite. Thus, parasites that grew and/or multiplied rapidly upon initial infection, then entered a phase requiring fewer resources (Type 1), would induce early fecundity reduction whereas parasites that exhibited a slow and sustained growth or multiplication requiring more resources as time progressed (Type 2) would induce later reductions in reproductive effort that may be accompanied by early fecundity compensation. Finally, parasites that had rapid and sustained growth and multiplication (Type 3) would cause early, and possibly permanent, fecundity reduction. Evolutionary pressures would select for adaptive strategies that maximise host fitness in the face of an infection that could not be eliminated. Thus, parasites may invoke a compensatory mechanism in their host that may also operate in other times of stress, such as starvation. For example, an immediate reduction in egg production would divert metabolic resources to somatic tissues and thereby provide reserves that enable the insect to survive until the metabolic demands of the parasite lessen.

4.3.4. A host–parasite amalgam

It can be argued that fecundity reduction would benefit both the parasite and the host (Hurd, 1998). Trophically transmitted parasites will gain if the host has sufficient resources to survive until the infection becomes patent. Likewise, parasites transmitted by haematophagous insects will only achieve transmission in a vector that has sufficient resources to be able to fly in search of a blood meal. In addition, the host or vector may gain by optimising its fitness via a temporary adjustment of resource allocation away from reproduction. If this allows the host to survive as long as, or even longer than, an uninfected host this may provide a period of catch up time for reproduction. Alternatively, reproduction may be traded off against defence when parasite virulence could otherwise cause early host death. Thus, phenotypes of infected hosts could be shared phenotypes, created by both the parasite and the response of the host to the presence of the parasite in what Wellnitz (2005) termed a 'negotiated settlement' and as was assumed in a model created by Restif and Koella (2003). If this benefits both symbionts then it will evolve rapidly and is likely to have more than one mechanism underlying the shift of resources.

4.3.4.1. A mafia-like strategy

Recently, the concept of a mafia-like strategy of manipulation has been revisited following its initial application to the response of birds to cuckoo's eggs in their nests (Ponton *et al.*, 2006). This hypothesis suggests that hosts may co-operate with manipulative parasites as a damage-limitation strategy to avoid additional costs that would be imposed by the parasite in the absence of compliance. However, there are no other examples of behavioural manipulation where greater costs have been shown to be associated with non-compliance. Crickets usually jump into water when infecting nematomorphs are mature, thereby enabling the hairworms to escape into the correct habitat for their breeding. When non-compliance was forced upon infected crickets, non-compliant females did not produce any eggs although some compliant ones did, suggesting a greater cost for non-compliance. However, compliance did not give infected crickets an overall fitness advantage over non-compliant ones as, although some eggs were produced, mating behaviour was disrupted in compliant females and males were completely castrated (Biron *et al.*, 2005c).

4.3.4.2. Host compensatory responses

Coercion, by imposing penalties for non-compliance, is not the only explanation for the involvement of both parasite and host genomes in the eventual phenotypic outcome of infection. Parasites may affect fitness-related host traits so that compensatory responses are stimulated in the host resulting in enhanced chances of parasite transmission (as discussed by Lefèvre *et al.*, 2008). In this respect, parasites can be regarded as influencers of the host phenotype, much as environmental conditions may influence it. This implies a degree of plasticity in host life-history traits that is well recognised, and may be particularly relevant to parasite-induced changes in reproductive fitness. As we have seen, hosts can down-regulate reproductive effort in the face of infection and thus alter resource management such that more effort may go into resistance or tolerance mechanisms, survival chances will be greater and both the parasites and the host will benefit. Compensatory responses could be initiated by the parasite if it mimics other intrinsic or extrinsic stimulators, or if it intervenes directly or indirectly in regulatory pathways.

4.4. MODELS FOR TESTING EVOLUTIONARY HYPOTHESES

Evolutionary biologists have provided us with several hypotheses to explain why hosts may respond to parasitic infection by making changes in life-history traits. How can we test whether any of these hypotheses

hold true for the particular association under investigation? Only by investigating the molecular, biochemical and physiological alterations that take place during infection can we begin to understand the mechanisms underlying trade-offs in life-history traits. This trail should lead us to identify which partner is driving these changes.

In 1998 it was recognised that, despite the numerous examples of parasites that disrupt some aspect of insect reproduction, there was no example in which the underlying mechanism had been fully elucidated (Hurd, 1998). Regrettably, this is still the case. The following associations have been investigated most fully and they serve to illustrate the complexity of mechanisms underlying changes in life-history traits and, in the first example at least, how experimentation has helped to determine which partner is driving a phenotypic change in the host.

4.4.1. Hymenolepis diminuta / Tenebrio molitor

4.4.1.1. A tapeworm driver

Embryonated eggs of the rat tapeworm, *H. diminuta*, are transmitted trophically to a variety of insects, including the darkling beetle *T. molitor*. If the egg shell has been cracked by the jaws of the beetle the onchosphere emerges in the gut, burrows through the mid-gut wall and, within approximately 15 days, develops into a mature metacestode in the haemocoel. Beetles eggs are produced continuously and first oviposited at about day 9 post-eclosion. When infection occurs on day 3 to 4 post-eclosion a greater number of eggs are initially oviposited but then, as the parasites mature, oviposition is retarded and the hatch rate of eggs is significantly reduced. Over a period of 30 days, a significant reduction in egg production occurs if beetles are kept in very crowded conditions (Hurd and Arme, 1986). Cole *et al*. (2003) also detected a significant increase in the number of eggs laid by infected 9-day-old females, but the mean number of larvae produced by infected beetles was reduced by approximately 35% by day 30 post-infection (Hurd, 1990).

Significant perturbations of several aspects of reproductive physiology that are indicative of changes in resource allocation have been recorded in this association. Briefly, oogenesis is retarded at two points of development: in the fat body, synthesis of the yolk protein, vitellogenin, is reduced (Webb and Hurd, 1996) and in the ovary, uptake of vitellogenin by developing oocytes is hindered. Each of these organs appears to be affected by molecules of different origin. When fat bodies from uninfected females were subjected to *in vitro* incubation followed by radioimmunoassay to detect vitellogenin, a significant density-dependent reduction in synthesis was detected if live metacestodes were present in the incubation medium. Extracts from metacestodes at an early stage of development (I–II) (Voge and Heynemann, 1957) had a far greater effect than those that had retracted

the scolex and whose growth was completed (Webb and Hurd, 1996). Thus, reproduction is down-regulated when the parasite is growing rapidly and exerting maximum metabolic demands upon its host.

When live parasites were replaced by various parasite extracts, the production of a parasite manipulator molecule(s) was confirmed (Webb and Hurd, 1999). Medium in which early-stage parasites had been incubated for 4 h was shown to reduce vitellogenin synthesis by 61% compared with tissue incubated in control medium (Hurd and Webb, personal communication). Properties of the molecule suggest that it is proteinaceous. Though originally thought to be 10–50 kDa in size (Webb and Hurd, 1999), reverse-phase high-performance liquid chromatography of acid extracts of stage II metacestodes has now revealed the presence of a peak containing two bioactive compounds. One of these molecules appears to be a short peptide of 200–250 Da, however, it has not been possible to obtain a sequence for this peptide, which appears to be end terminally blocked or cyclic in nature (Webb *et al.*, personal communication).

Investigations of the mode of action of this manipulator molecule have revealed that impairment of vitellogenin synthesis in infected beetles is not due to a reduction in the level of transcript of the single copy *T. molitor* vitellogenin (Vg) gene. Contrary to expectation, Vg messenger RNA (mRNA) abundance was significantly increased in fat body tissue dissected from infected beetles from 6 days post-infection onwards. *In vitro*, live stage I–II metacestodes cultured with fat body from uninfected females produced a highly significant elevation in Vg gene transcript whereas stage V–VI parasites had no effect (Warr *et al.*, 2006b). One explanation for this apparent contradiction between an elevation in gene transcription and a decrease in protein synthesis is that the tapeworm manipulator molecule alters the storage profile of Vg mRNA, with fewer transcripts being translated than produced. A positive feedback loop, which responds to falling Vg synthesis by increasing gene transcription, would account for the increased transcription. This could result in a permanent change in transcription, thus explaining why levels are higher *in vivo*, even when the parasite has matured.

It is possible that the manipulation molecule produced by *H. diminuta* mimics the action of an endogenous insect hormone or peptide that controls vitellogenin synthesis. Juvenile hormone (JH) is thought to be involved in the control of *T. molitor* vitellogenesis (Laverdure, 1970) and has been shown to contribute to Vg gene transcription regulation in other insects (Wyatt and Davey, 1996). Circulating titres of JH are higher in infected females very early in infection but not at later times (Cole *et al.*, 2003) thus it is possible that an early JH spike could up-regulated transcription. However, there is some evidence that, in locusts, vitellogenin synthesis may be controlled at the post-translational level by the action of

adipokinetic hormone (AKH; Moshitzky and Applebaum, 1990; Glinka *et al.*, 1994). Nematodes are known to produce peptides with AKH-like activity (Davenport *et al.*, 1991). Interestingly AKH is also involved in the regulation of carbohydrate metabolism in insects (Gade and Auerswald, 2003), another aspect of physiology affected by tapeworm infections in *T. molitor* (Kearns *et al.*, 1994). Until the identity of the tapeworm manipulator molecule is determined, it is not possible to say whether its mode of action is to mimic an endogenous regulator.

4.4.1.2. A beetle driver

It is worth noting that there may be an additional mechanism involved in the down-regulation of vitellogenesis. Trophocytes from the fat bodies of infected females undergo apoptosis with a positive relationship between burden of infection and the number of apoptotic cells being noted. Apoptosis is rarely seen in the fat bodies of uninfected females and it could not be induced when fat body tissue from uninfected females was incubated with live metacestodes, suggesting that the parasite manipulator molecule is not inducing cell death but that this is driven by host factors (Warr *et al.*, 2006a).

Vitellogenin, secreted into the haemolymph from the fat body, is taken up by the oocytes developing in the terminal follicles of the ovaries by receptor-mediated endocytosis. To gain access to the oolema, the yolk protein passes between the cells of the follicular epithelium via gaps that develop when follicle cells shrink due to the loss of water following JH activation of a membrane-bound Na^+/K^+ ATPase (Davey *et al.*, 1993). This process leads to the development of patency, which has been linked to the vitellogenic status of follicles in *T. molitor*. The development of patency is retarded in *H. diminuta*-infected beetles as a result of a disruption in the binding of JH III to follicle cell microsomal binding sites early in infection. Binding recovers later and can be rescued by the application of 50-nM JH III. Scatchard analysis revealed a five-fold higher dissociation constant (K_d) value for infected beetles; indicative of the presence of a competitive binding inhibitor (Webb and Hurd, 1995a,c).

The retardation/inhibition in the development of patency hinders the uptake of vitellogenin by the oocyte and this is reflected in the level of vitellin in developing follicles of various sizes that are present early in infection. Here too, by day 15 post-infection (when parasites are mature), vitellin uptake appears to have recovered. The reduction in vitellin content in developing follicles is reflected in a highly significant accumulation of vitellogenin in the haemolymph of infected beetles, despite the reduction in fat body synthesis (Webb and Hurd, 1995b).

Although follicle resorption is rarely seen in uninfected beetles, Warr and colleagues (2004) observed that by day 15 post-infection over 15% of follicles were resorbing and that this figure reached over 30% by 30 days

post-infection. This must contribute to the decline in longer-term reproductive potential observed in infected *T. molitor*.

Both male and female *T. molitor* can act as hosts for *H. diminuta* thus any manipulator molecule secreted into the haemolymph would be expected to be present in haemolymph from both sexes. By transferring haemolymph from donor-infected beetles into uninfected females, Major and colleagues (1997) demonstrated that, although haemolymph from females with early-stage metacestodes significantly reduced the vitellin content of follicles, haemolymph from infected males did not. It was suggested that, in contrast to the direct effect that the parasites have on vitellogenin synthesis, the host itself is driving changes in the ovary. The female may be producing an inhibitor molecule that acts by competitively binding to JH receptors on the follicular epithelium, thus slowing down the uptake of vitellogenin early in infection. The nature of this molecule is unknown but it is feasible that it may be produced in response to several stressors. It would act by diverting resources away from egg production, as has been documented for schistosomin in a trematode/snail model (de Jong-Brink, 1995) (see Section 4.4.5.3).

In summary, there is evidence from this association that the parasite is producing a manipulator molecule that directly suppressed the direction of resources to host reproductive effort but also that the host is responding to infection by regulating its own egg production.

Metacestode-induced fecundity reduction at a period of rapid parasite growth is likely to benefit both parasite and host if resources are directed away from reproduction with a concomitant increase in host life span. This is indeed what happens. A comparison was made of survivorship in eight populations of infected and uninfected male beetles and eight populations of infected and uninfected females. No beetles died during the first 8 days post-infection, during which time the metacestodes were growing rapidly and vitellogenesis was affected. Mortality increased steadily from day 12 onwards in all populations, with half the uninfected male beetles dying by day 18 and half the uninfected females by day 22. In contrast, there was a highly significant increase in median survival time to 50% mortality in the infected female groups (8 additional days) and a lesser, but still significant increase of 4 days in the infected male groups. Overall there was a hazard ratio of 2.35 (control vs infected) (Hurd *et al.*, 2001). Interestingly it is in the females that we see a diversion of resources away from reproduction. Some aspects of male reproduction are enhanced (Carver and Hurd, 1998) and some reduced (Hurd and Ardin, 2003) by infection and life span is not increased so much as in the female. As female beetles continue to lay eggs throughout their life span, they should have an opportunity to compensate for the decrease in vitellogenin synthesis and ovarian uptake that occurs when the parasites are growing rapidly. Importantly, the increase in life span will also increase the parasite's opportunities for trophic transmission.

4.4.2. Malaria-infected mosquitoes

Protozoans of malaria parasites of the genus *Plasmodium* are transmitted from host to host by mosquito vectors. Gametocytes that are imbibed with a blood meal rapidly differentiate into gametes. Fertilisation takes place within the mid-gut lumen and a motile zygote, the ookinete, develops within 24 h, traverses through the mid-gut epithelium and transforms into an oocyst below the basal lamina. Within 2–3 weeks, sporogony takes place and sporozoites are released into the haemocoel and invade the salivary glands prior to transmission to the next host (Baton and Ranford-Cartwright, 2005). During this time, the vector may have undergone three to four gonotrophic cycles, each one initiated by a blood meal and resulting in the oviposition of a batch of eggs. Gonotrophic cycles are completed and eggs are laid in 3–4 days post-blood feeding; with transcription of vitellogenin genes being massively up-regulated within hours of blood feeding and vitellin deposited in oocytes of all terminal follicles simultaneously.

Several species of malaria have been shown to significantly reduce the fecundity (number of eggs) and fertility (larvae produced) of anopheline and aedine mosquitoes (see review by Hurd, 2003 and see Gray and Bradley, 2006; Marrelli *et al.*, 2006). In laboratory models, reduced egg production occurs when infections are initially established and also later in infections, when oocysts or sporozoites are present (Hurd, 2003). Interestingly, blood meals containing *Plasmodium falciparum* gametocytes that were non-infective to mosquitoes increased the number of females that developed a batch of eggs (gravidity) following the meal, even though blood meal size remained unchanged (Ferguson *et al.*, 2003).

The rodent malaria *P. yoelii nigeriensis* has been used as a model to investigate the mechanism underlying fecundity reduction in *Anopheles stephensi* and *A. gambiae*. As with the tapeworm/beetle model, several aspects of vitellogenesis are disrupted by the presence of the parasites, though via different mechanisms. These are reviewed in detail by Hurd (2003). In the fat bodies of *A. gambiae*, there is a slight reduction in the abundance of Vg mRNA 36 h post-feeding, when ookinetes have just penetrated the mid-gut epithelium and are transforming into oocysts. A significant reduction in Vg mRNA is associated with infection throughout a second gonotrophic cycle, when oocysts are present (Ahmed *et al.*, 2001). Circulating titres of vitellogenin are significantly lowered by 30 h post-infection but 24 h after a second blood meal vitellogenin begins accumulating in the haemolymph of infected females as a result of decreased sequestration into the ovary (Ahmed *et al.*, 2001). In common with the *H. diminuta/T. molitor* model, development of patency of the follicular epithelium is retarded and a significant number of follicles are resorbed. However, in mosquitoes this is as a result of apoptosis of

patches of the follicular epithelial cells (reviewed by Hurd, 2003). These events do not appear to be dependent upon parasite density and there is no evidence that they are directly induced by molecules secreted by the parasites. However, circumstantial evidence suggests that they may occur as a result of the induction of an immune response to infection.

The evolutionary consequences of malaria-induced fecundity reduction may be similar to those proposed for the tapeworm-infected beetle in that a change in resource management may provide sufficient reserves for an infected mosquito to survive to take another blood meal, mature another batch of eggs and transmit sporozoites. If so, then both partners may gain but the effect of malaria upon mosquito longevity is still controversial (Ferguson and Read, 2002) and investigations need to be conducted in field situations where environmental stresses are also at work.

4.5. INDIRECT MECHANISMS UNDERLYING FECUNDITY REDUCTION

4.5.1. The immune system

Although parasitised insects have failed to avoid or totally eliminate an infection, the parasites may have been detected by the defence surveillance system, with a resultant up-regulation of the effector arm of the immune system. Traditional life-history theory assumes that defence systems are costly (Sheldon and Verhulst, 1996) and the field of ecological immunology is replete with literature discussing possible fitness costs of immune defence and the trade-offs that might operate between defence and other life-history traits. However, this over-riding assumption has been questioned. Costs may depend upon a variety of factors such as the mechanism of defence, the condition of the host, environmental variables, and variation within parasite and host species (Coustau *et al.*, 2000; Rigby *et al.*, 2002; Sandland and Minchella, 2003; Schmid-Hempel, 2003).

Costs have been linked to insect immune defence systems by comparing the fitness of strains that are resistant or susceptible to infection, as discussed by Hurd and colleagues (2005) and seen in the recent description of *Drosophila nigrospiracula* artificially selected for resistance to the ectoparasitic mite *Macrocheles subbadius*. Selected flies exhibited reduced fecundity that was correlated with increase resistance, a trait that was increased with increasing temperature (Luong and Polak, 2007).

Costs have also been demonstrated by artificial stimulation of the immune system by injection of xenobiotics such as Sephadex beads or immune elicitors such as LPS (Moret and Schmid-Hempel, 2000; Schwartz and Koella, 2004) but few studies have demonstrated a mechanism that links a defence response to infection with changes in reproductive success.

One such study is the mosquito/malaria association outlined in Section 4.2. Humoral activity against Gram-positive bacteria was induced in a dose-responsive manner following LPS injection into female mosquitoes immediately after they had been blood fed. A concomitant reduction of approximately 50% in the amount of protein accumulated by developing follicles occurred, and LPS injection significantly reduced egg production (Ahmed *et al.*, 2002). Both LPS injection and the induction of a melanisation response using Sephadex beads was shown to cause apoptosis in cells of the ovarian follicular epithelium, indicating that the mechanism underlying reproductive costs associated with immune stimulation are the same as those that operate in malaria infections.

Within a few hours of an infective-blood meal, malaria parasites induce an immune response in anopheline mosquitoes. Initially this consists of the up-regulation of inducible nitric oxide synthase and the subsequent production on nitric oxide by L-arginine oxidation. Luckhart and co-workers (e.g., see Luckhart *et al.*, 2003) have uncovered a complex series of reactions controlling this response that involve signalling between factors from the mammalian host, the parasite and the mosquito. Luckhart suggests that the insulin-signalling cascade plays a central role in the regulation of reproduction, ageing and nitric oxide synthase expression (Luckhart and Riehle, 2007). In addition to nitric oxide synthase, *Plasmodium* alters the transcript of a number of immune-associated genes, including anti-microbial peptides and elements of the phenoloxidase cascade associated with melanisation (reviewed by Whitten *et al.*, 2006).

One explanation for commonality in the mechanism of egg resorption associated with infection and immune stimulation is that *Plasmodium* may induce a compensatory response in the mosquito that operates via the immune system. Thus, a scenario can be envisaged in which an invading parasite stimulates an immune response and, in doing so, engenders a compensation response in the host that operates via immune genes and/or signalling cascades that have pleiotropic effects; a positive effect on one trait (defence) and a negative effect on another (reproduction). The resultant curtailment of egg production will release resources that benefit the parasite and the host. Furthermore, this response may not be specific to *Plasmodium* infection but operate in the face of a variety of challenges by parasites and pathogens. There is clearly scope for more investigation of this model of parasite-induced fecundity reduction to test this hypothesis.

4.5.2. Neuromodulators

An alternative way in which trade-offs between life-history traits can be modulated is via the insect nervous or endocrine system. Although there are no studies that demonstrate unequivocal links between infection,

neuro/endocrine modulators and reproduction, evidence from the *H. diminuta/T. molitor* model outlined in Section 4.4.1.2. suggests these putative pathways are worthy of further investigation. Here again, there is scope to examine different parasite–host associations to determine whether the parasite is producing manipulation molecules that directly interact with, or mimic, aspects of pathways that control host reproduction or whether the host itself is doing the regulating. After examining evidence from three parasite–invertebrate associations, Adamo (2002) concluded that it may be too expensive for the parasite, with its small biomass relative to the host, to produce sufficient neuromodulator molecules to affect a change in the host. A more likely scenario is for the host to produce neuromodulators in response to infection. Clearly, parasite modulator molecules would have to be very potent. *H. diminuta* metacestodes may be producing such a molecule (see Section 4.4.1.1).

If an insect is directly regulating its own reproduction in response to infection, it must be receiving signals concerning its infection status. This again brings into play a likely role for the immune system in initiating the orchestration of life-history changes.

4.5.3. Links between parasites, the insect immune system, neuroendocrine modulators and reproduction

These connections have been cleverly unravelled in *Lymnaea stagnalis* snails infected with the trematode *Trichobilhazia ocellata.* Depending on the age of the snails when initially infected, egg laying is reduced or ceases altogether. Originally thought to be of parasite origin, a cytokine-like molecule, schistosomin, is released by cells of the snail immune system. This signal molecule has several functions, including alteration of the electrophysical activity of a group of neuroendocrine cells, the caudodorsal cells, which regulate egg laying and competitive inhibition of calfluxin receptors involved in regulation of accessory gland secretions (reviewed in de Jong-Brink, 1995).

Although these three-way links have been little studied in parasitised insects, bacterial infections and/or non-pathological immune challenge are beginning to shed light on potential connections. A review by Adamo (2007) highlights links between insect nervous and immune systems involved in the induction of behavioural fever, anorexia and reproductive behaviour in response to infection. She discussed a possible role for biogenic amines such as octopamine (which can alter haemocyte activity), vertebrate-like cytokines, nitric oxide and eicosanoids in the mediation of a neural-immune connection, but concluded that variability across insect species may preclude the construction of generalised hypotheses.

We clearly need a much better understanding of the molecular, biochemical and physiological mechanism underlying modification in host

life-history traits. With respect to the pleiotrophic nature of life-history traits and the molecules involved in modulating these switches, promising headway is now being made via the study of changes in host proteomes (Biron *et al.*, 2005a,b; Lefèvre *et al.*, 2006, 2007a,b) and genomes (e.g., Christophides *et al.*, 2004). In *Drosophila*, specific genes have been identified that confer antagonistic pleiotrophy on life-history traits. For example, mutant flies with disrupted insulin-like receptors live longer that wild-types, but their reproduction is reduced (Tatar *et al.*, 2001) and over-expression of a heat-shock protein reduces fertility but increases longevity (Silbermann and Tatar, 2000).

4.6. LIFE-HISTORY TRAITS IN AN ECOLOGICAL SETTING

Vertebrates are known to respond to environmental stressors such that life-time fitness is enhanced. These responses may be physiological or behavioural, can occur rapidly and result in an 'emergency life-history stage' that may be temporary (Wingfield, 2003). It is likely that insects respond in a similar manner. This clearly adds another dimension to life-history trade-offs that may result from infection. One trade-off that is well documented in many systems is stress-related immunosuppression.

Exposure of the cricket *Gryllus texensis* to several fight-or-flight stressors demonstrated that physical exercise (running for 15 min) resulted in an immediate, but short lived, increase in susceptibility to the opportunist Gram-negative bacterium *Serratia marcescens*. Agnostic behaviour between pairs of males previously isolated for 3 days also increased the susceptibility of both winners and losers, as did exposure to extreme heat (35 °C for 15 min) but not cold (4 °C for 15 min). Immune function in the form of lysozyme-like activity was significantly reduced following restraint and also heat treatment (Adamo and Parsons, 2006).

Response to stress is mediated via inducible signalling molecules, the cytokines. In invertebrates, mammalian-like signal molecules in the form of opioids are employed in response to stress (Stefano *et al.*, 2002) and may provide the link between stress and immunosuppression. Octopamine injection increased the susceptibility of *G. texensis* to *S. marcescens* (Adamo and Parsons, 2006). However there are contradictory reports concerning the role of octopamine in insect immune functioning and more studies in a range of insects are required before this can be resolved. Adamo and Parsons (2006) also suggest that endocrine signalling may realign multi-functional compounds, giving the example of the carrier molecule apolipophorin III, which is involved in immune functioning as well as energy mobilisation, but may not be able to perform both functions simultaneously.

4.6.1. Measuring the effect of environmental stresses

Parasite-induced fitness costs are usually measured in the laboratory, where only context-dependent fitness costs may be observed. For most associations we have little idea of the relative importance of parasitism on host fitness within a fluctuating environmental setting with multiple stressors present (Thomas *et al.*, 2002; Tripet *et al.*, 2008). Ideally, investigations should be conducted in the field. Here additional stressors likely to be operating on the infected insect may be biotic, such as predation and competition, or abiotic, such as desiccation, limited resources or the presence of insecticides.

Unfortunately, the effects of environmental stressors on the modulation of reproduction in infected insects have rarely been investigated (but see Section 4.4.3.4 and Hurd *et al.*, 2005). It is difficult to simulate the complexity of an environmental setting in the laboratory and studies in the field have limited value if the history of the insects under study is unknown. However, the large-caged environments being developed for trials of genetically engineered insects (Benedict *et al.*, 2008) may provide a logistical solution to these problems.

4.7. CONCLUSIONS

Parasites can have profound effects on host life-history traits such as reproductive fitness. Although this review has been confined to insect infections, there are parallels to be made both with other invertebrate/parasite associations and with vertebrate hosts. In the past, there has been a tendency to view these changes either from the standpoint of the parasite or from the host. However, it has been argued here that both symbionts could be driving these changes as they may both stand to gain by altering host reproductive output.

The key requirement for testing theoretical models and hypotheses concerning parasite–host evolution is the elucidation of molecular, biochemical and physiological mechanisms underlying the changes in phenotype that we can observe. Information gained from laboratory models will be enhanced by studying insects and parasites that have co-evolved and by observing interactions in a natural setting, but currently we have to rely on information from very few model systems.

If parasite molecules that directly manipulate the host's life-history strategies can be identified, these will clearly point to selection pressures acting upon this partner whereas a clear-cut host response to the presence of the parasite would point to the pressure being upon the host. However, evidence from a beetle/tapeworm model demonstrated that fecundity reduction is driven by both hosts and parasite and we do not yet have

enough information about malaria-infected mosquitoes to make any judgements. The tapeworm is a metazoan parasite, but even so, the parasite biomass required to induce a change in host vitellogenesis is very small, suggesting that this must be an extremely potent molecule. The likelihood of protozoans producing enough manipulator molecules to have critical affects on the host may be dependent on the intensity of infection. The possibility that parasites are causing hosts to change life-history strategies via molecules involved in immune signalling is an attractive proposition that is gaining support from studies of a few parasite–host associations, including malaria-infected mosquitoes. A proteomics approach to investigating these questions is a promising way forwards.

The objectives of exploring the molecular basis for insect–parasite interactions in a natural setting sets exciting challenges for those interested in understanding the evolutionary pressures that have moulded the complex interactions that take place between parasites and their hosts. Moore (2002) ends her book on parasites and animal behaviour with the comment that our knowledge exceeds our understanding. Currently this observation is equally apt when applied to parasite-induced alteration of host life-history traits such as reproductive fitness. Hopefully, this will not be the case for much longer.

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CHAPTER 5

Ecological Immunology of a Tapeworms' Interaction with its Two Consecutive Hosts

Katrin Hammerschmidt* and **Joachim Kurtz†**

Contents

Abstract

Host–parasite interactions in parasites with complex life cycles have recently gained much interest. Here, we take an evolutionary ecologist's perspective and analyse the immunological interaction of such a parasite, the model tapeworm *Schistocephalus solidus*, with its two intermediate hosts, a cyclopoid copepod and the

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three-spined stickleback. We will be focussing especially on the parallel links between the different phases during an infection in the different hosts; the immunological interactions between host(s) and parasite; and their impact on parasite establishment, growth, host manipulation and parasite virulence in the next host in the cycle. We propose to extend the 'extended phenotype' concept and not only include the ultimate but also the proximate, physiological causes. In particular, parasite-induced host manipulation is suggested to be caused by the interactions of the parasite with the hosts' immune systems.

5.1. INTRODUCTION

Life cycles of the majority of parasites, like protozoans, nematodes, trematodes, cestodes and acanthocephalans are usually complex: they require two, or more, consecutive invertebrate and vertebrate hosts. While our understanding of the evolution of complex life cycles is currently still in its infancy (Parker *et al.*, 2003a), parasitological knowledge on the interaction of parasites with their hosts is more comprehensive. However, most studies have focused almost exclusively on the interaction of the parasite with one of the hosts, usually the vertebrate. In stark contrast, knowledge is rather meagre when it comes to how parasites interact with both hosts, often due to the lack of studies addressing the invertebrate, intermediate host. Recent theoretical and empirical studies on multi-host cycles even indicate that parasite virulence and fitness in the final (vertebrate) host can only be fully understood if the interactions between parasites and their intermediate (usually invertebrate) hosts are taken into account (Ebert, 1998; Gandon, 2004; Mackinnon and Read, 2004; Parker *et al.*, 2003a).

Among others, one key factor proposed to drive parasite virulence in multi-host systems is the degree of similarity between the invertebrate and vertebrate hosts' immune system. This might lead to a positive correlation in the parasite's ability to exploit the different hosts (Gandon, 2004). Models such as *Plasmodium* have proven to be successful candidates for investigating a parasite's interaction with the immune systems of both hosts (Garver *et al.*, 2008; Luckhart *et al.*, 1998; Sobolewski *et al.*, 2005). However, there are also limitations. For example, laboratory infections normally make use of non-natural hosts (such as mice instead of humans) and therefore, systems that have not had a chance to co-evolve naturally. Further, invertebrate vector species are highly diverse and possess diverse immune strategies to control their various parasites (Loker, 2004). This highlights the need to broaden our knowledge of other multi-host–parasite model systems, with a special focus on naturally co-evolved host–parasite systems and the immune interactions of parasites with *all* hosts.

The present article will review recent studies on the tapeworm *Schistocephalus solidus*. This is a parasite with a complex life cycle, which is developing into an established model for addressing basic evolutionary and ecological questions of host–parasite interactions. We will employ an ecological immunological approach, and focus on the causes and consequences of variation in immune function of hosts against the parasite (Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; Sheldon and Verhulst, 1996). Interactions between the parasite and its different consecutive hosts will be analysed and linked to parasite fitness (i.e., number of produced offspring), which can be estimated from body size in *S. solidus* (Schärer *et al*., 2001). We will also take an evolutionary biologist's viewpoint and analyse the interaction of the parasite with its hosts with regard to the scope for co-evolution (Webster *et al*., 2007; Woolhouse *et al*., 2002). Host–parasite co-evolution is defined as the reciprocal genetic adaptation in host and parasite. A necessary prerequisite for co-evolution to take place is a genetic variation among host and parasites in their ability to exploit each other. The question of whether particular steps of the interaction between the parasite and its hosts show genetic specificity will be addressed, and whether this could be subject to co-evolutionary change.

5.2. THE MODEL PARASITE *SCHISTOCEPHALUS SOLIDUS*

The cestode *S. solidus* has become a model system for investigating host–parasite interactions involving a complex life cycle. This pseudophyllidean tapeworm has been studied in the laboratory since 1946 (Smyth) and methods for culturing and manipulating have been constantly improved since then (Dubinina, 1966; Kurtz, 2003; Wedekind, 1997). *S. solidus* has to pass through two intermediate hosts, a cyclopoid copepod and the three-spined stickleback *Gasterosteus aculeatus*, before it reproduces in its definite host, any fish-eating bird or mammal. When eggs are released into freshwater with the bird's faeces, coracidia, the first (free-swimming) larval stage, hatch (Fig. 5.1). These are preyed upon by various species of cyclopoid copepods, although we will mainly refer to *Macrocyclops albidus* (Fig. 5.2). This is a large species of freshwater copepod that has been used in many of the experimental studies reviewed here. After ingestion and successful infection, tapeworms develop into the second, the procercoid stage (Fig. 5.1). The procercoid grows in the copepod until it is infective for the next host, the three-spined stickleback. Here it transforms to a plerocercoid and grows in the stickleback's body cavity until infective to the definite host (Fig. 5.3). *S. solidus* is perhaps one of the best model systems for studying a macro-parasite with a complex life as:

(i) it is one of very few naturally occurring (thus, likely to have co-evolved) host–parasite systems studied (many others include non-natural hosts);

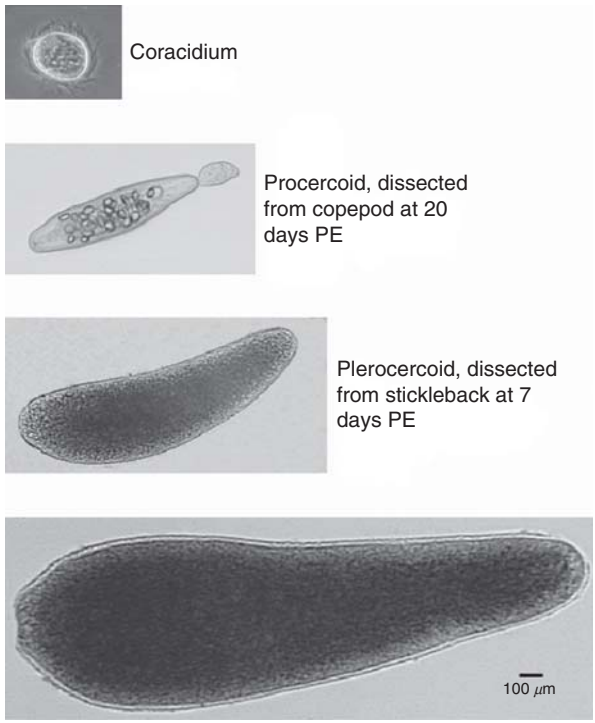

FIGURE 5.1 Developmental stages of *S. solidus*: the free-living coracidium, the procercoid in the copepod and the plerocercoid in the stickleback. The size differences between the stages (the scale is the same for all stages). The mature stage is not shown, as it is more than 4,000 times the size of the plerocercoid after 7 days in the stickleback. PE, post-exposure.

(ii) both intermediate hosts and all stages of the parasite can easily be kept and manipulated in the laboratory (the definite host can successfully be replaced by an *in vitro* breeding system) (Smyth, 1946; Wedekind, 1997);
(iii) the course of infection of the first host, the copepod, can be traced non-invasively, which is quite unique for a macro-parasite (Kurtz *et al.*, 2002) (Fig. 5.2);
(iv) the complete genome sequence of the second intermediate host, the three-spined stickleback is available, facilitating genetic analyses of traits involved in the host–parasite interactions and immunity;
(v) *S. solidus* is widespread in the Northern Hemisphere and is easily accessible (restricted by the range of three-spined sticklebacks). This ensures the use of animals that are not already adapted to laboratory conditions for several generations;

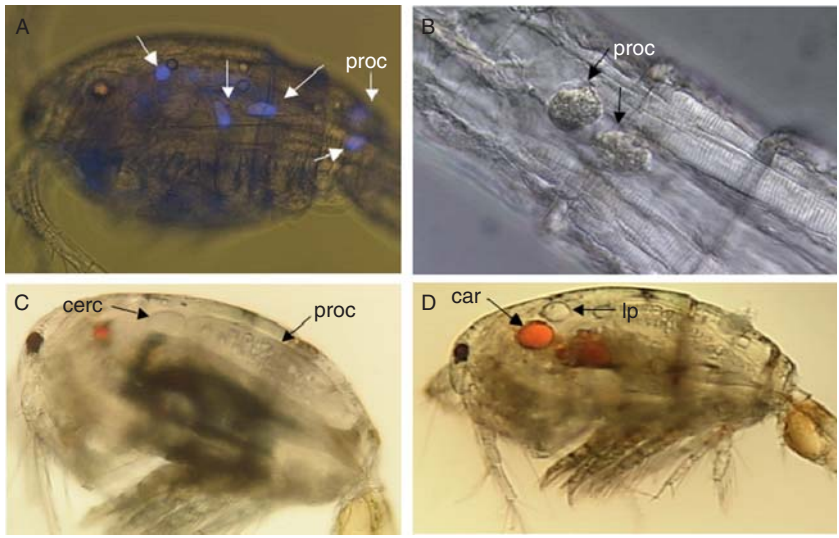

FIGURE 5.2 The course of a *S. solidus* infection in the copepod can be monitored non-invasively. (A) An early infection of *S. solidus* can be visualised inside the copepod by labelling coracidia with fluorescent vital dyes such as the blue 7-amino-4-chloro-methylcoumarin (CMAC), which is retained during the development to the procercoid (proc) (modified from *Kurtz et al., 2002*). (B) Dead, early-stage, procercoids in the copepod. (C) A male *M. albidus* copepod with a fully developed *S. solidus* procercoid; the cercomer (cerc) is clearly visible. (D) Quantity of carotenoids (car) and other lipids (lp) can be easily monitored.

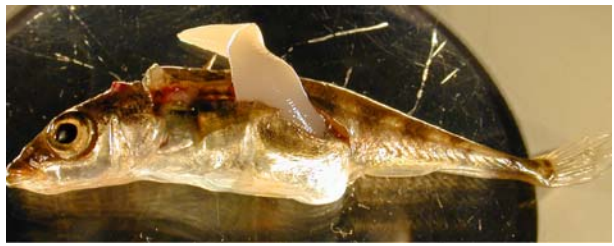

FIGURE 5.3 Adult *S. solidus* leaving a dissected three-spined stickleback. Picture © M. Kalbe, reproduced with permission.

(vi) it is extremely variable in its life history strategies among populations (facilitates understanding through intra-population comparisons);
(vii) current knowledge on various aspects of the host–parasite relationship is available, particularly morphology (e.g., Chubb *et al.*, 1995; Hopkins and Smyth, 1951), physiology (e.g., Smyth, 1946, 1954),

evolutionary ecology (e.g., Jäger and Schjørring, 2006; Wedekind *et al*., 1998) and behaviour (e.g., Barber and Huntingford, 1995; Wedekind and Milinski, 1996).

Recent studies have also started to investigate the immune interactions between the two intermediate hosts and the respective parasite stages (Hammerschmidt and Kurtz, 2005a; Kurtz, 2007; Kurtz and Franz, 2003; Kurtz *et al*., 2004; Scharsack *et al*., 2004, 2007). Both immune defence systems of the invertebrate and vertebrate host consist of similar and differing parts. Thus, we could expect that parasite antigens might be detectable by the invertebrate immune system, the vertebrate immune system or both. As an invertebrate, the copepod will mainly rely on innate defences. These are characterised by pattern recognition receptors detecting conserved pathogen-associated molecular patterns of parasites (Hoffmann *et al*., 1999; Janeway and Medzhitov, 2002). Although such components of innate immunity are expected to be unspecific, in that they are unable to differentiate among parasite genotypes, recent evidence suggests that invertebrates have evolved also more specific immunity (see below). The stickleback possesses innate and adaptive defences. The innate immunity of vertebrates consists of both evolutionarily conserved parts, which show homology with the invertebrate innate defences, as well as possessing traits that evolved later and are therefore unique to vertebrates. The adaptive (or acquired) immunity of vertebrates is characterised by the somatic diversification of the genes encoding for T-cell receptors and antibodies, leading to the ability to establish a highly specific immune memory (Janeway *et al*., 1999).

This review will discuss host–parasite adaptations with a special focus on immunological processes against a background of morphological, behavioural and evolutionary aspects and thus provide an integrated picture of a macro-parasite adapted to a multi-host life cycle. We will concentrate on the interaction between *S. solidus* and its two intermediate hosts, the copepod and stickleback, as the co-evolutionary interaction can be expected to be tightest here. Parasite growth and resource acquisition occur in these two intermediate hosts, such that the effects on host fitness and therefore selection for host immune defences and parasite counter-defences are expected to be strong. By contrast, the fitness of the bird definitive host is expected to be rather unaffected by the tapeworm, as in contrast to other tapeworms, it does not attach to the host's intestine (Dubinina, 1966) and it is usually localised in the posterior portion of the birds intestine. Here, nutrients (such as carbohydrates) are scarce, which does not affect *S. solidus* as it does not grow, and its storages of up to 51% of glycogen (Hopkins, 1950) enables it to mature and even survive in completely nutrient-free media (Smyth, 1950). Due to the brief period (up to 5 days), it spends in the intestine, accumulation of the

tapeworms is rare so that significant pathogenic effects can be excluded (Dubinina, 1966).

Host–parasite interactions are shaped by the genetic and phenotypic traits of both hosts and parasites, and the outcome depends on several successive phases of defence and attack on both sides, as summarised in the defence component model (Schmid-Hempel and Ebert, 2003). We applied this concept to the *S. solidus* system and identified the key steps during its life cycle from the perspective of the parasite. Phase I: ingestion, infection and establishment, phase II: resource acquisition and immune evasion, and phase III: host switch and manipulation (Fig. 5.4). We will focus especially on these phases during an *S. solidus* infection, draw parallels between the invertebrate and vertebrate host and link the performance in both hosts to parasite virulence and fitness.

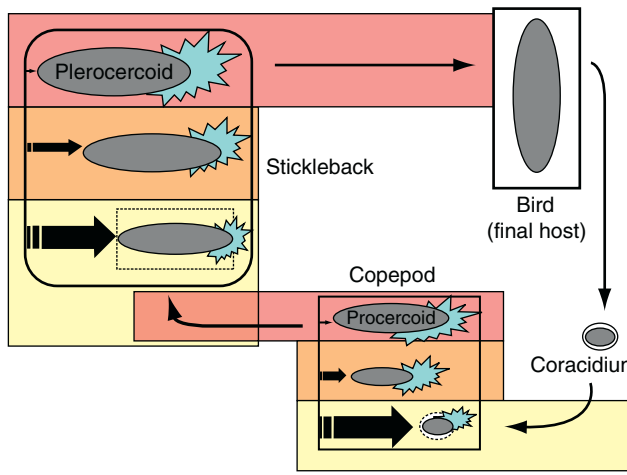

FIGURE 5.4 Schema of the life cycle of *S. solidus* with focus on the host–parasite interactions in its two intermediate hosts, the copepod and the three-spined stickleback. The interaction with each host can be divided into different successive phases, which are represented by the coloured areas: phase I (light grey): ingestion, infection and establishment; phase II (medium grey): resource acquisition and immune evasion; and phase III (dark grey): host switch and manipulation. During each phase, parasite size (here represented by differently sized parasites) and impact on each host (here represented by differently sized blot-like symbols next to the parasites) increases, whereas host impact (➔) on the parasite decreases. Only during phase I does the host seem to be able to clear an infection with *S. solidus*. During phase II and III, the interaction is dominated by the parasite, and at the optimal transfer time, the host functions merely as a 'transport vessel' to the next host in the cycle.

5.3. PHASE I: INGESTION, INFECTION AND ESTABLISHMENT

Before establishment, the parasite has to overcome at least three successive lines of host defence: (i) behavioural resistance, (ii) the mechanical and immunological barrier of the gut wall and (iii) immunity in the body cavity.

5.3.1. Behavioural resistance

The first line of host defence against the parasite is the avoidance of parasite ingestion (Hart, 1997), which has been reported for other host–parasite systems (e.g., Hulscher, 1973). For *S. solidus*, however, it has been shown that neither the copepod nor the stickleback host are able to avoid ingesting *S. solidus* (van der Veen and Kurtz, 2002; Wedekind and Milinski, 1996). In laboratory experiments, only 16% of the copepods failed to consume any of the offered coracidia (van der Veen, 2003) and infected copepods are rarely left over after experimental exposure to sticklebacks (unpublished observation). As encounter rates between copepods and coracidia are most probably low in most natural water bodies and as coracidia closely resemble ciliates, selection for recognition and avoidance of the parasite is presumably too weak for it to evolve (van der Veen, 2003). This situation is different in the stickleback, as prevalence of *S. solidus* can be very high in some populations (e.g., Arme and Owen, 1967; McPhail and Peakock, 1983; unpublished observation). Nevertheless, sticklebacks seem to be unable to resist eating infected copepods (see Wedekind and Milinski, 1996 for detailed discussion). If the stickleback's discriminatory abilities are such that parasite avoidance would imply that no prey of that size class or type would be eaten, then the costs of such a behaviour might be higher than the risk of infection (van der Veen, 2003).

5.3.2. Mechanical and immunological barrier of the gut wall

In both intermediate hosts, *S. solidus* has to migrate from the gut into its final location in the body cavity (Fig. 5.5). This process seems to occur relatively quickly. The first *S. solidus* procercoids appear in the haemocoel of the copepod in less than 3 h (1 h: Hammerschmidt, unpublished observation) post-exposure (PE) (Kurtz *et al.*, 2002). In sticklebacks, the first plerocercoids were found 14 h PE in the body cavity (Hammerschmidt and Kurtz, 2007). The time it takes to reach the final site in the stickleback is thus fairly small, as dead parasites were already found in the intestine 18 h PE (Hammerschmidt and Kurtz, 2007). Time is probably also a limiting factor for the parasite in copepods, as coracidia are short-lived (Dubinina, 1966; unpublished observation). Despite the relatively short

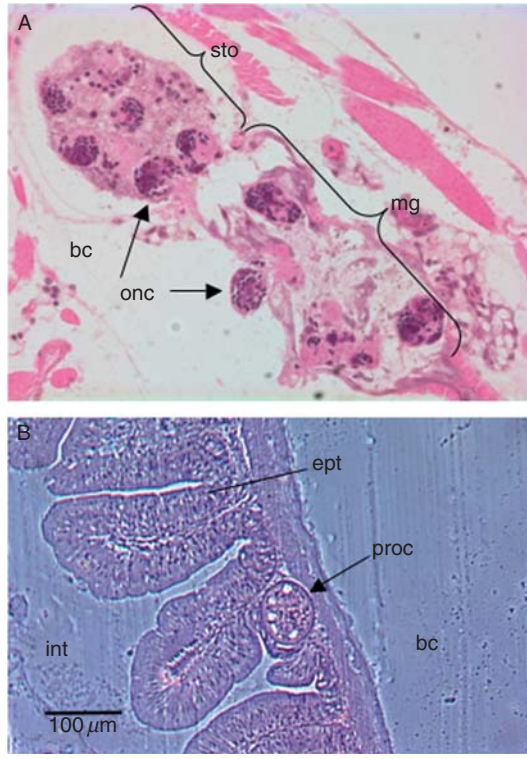

FIGURE 5.5 Passage from the gut into the body cavity of (A) the copepod and (B) the stickleback host. In (A), several oncospheres (onc) can be seen in the stomach (sto), whereas others already reached the mid-gut (mg), or are penetrating through the gut-wall into the copepod's body cavity (bc). In (B) a procercoid (proc) migrates through the stickleback's gut-wall from the intestine (int) into the body cavity (bc); here the epithelial layer (ept) has already been crossed.

time period for reaching the final sites in both hosts, there is evidence to suggest that this is the (only) time when the host is able to block off large numbers of parasites. Once individuals of *S. solidus* have entered the copepod or stickleback body cavity, none of the hosts seems able to eliminate the parasite by an efficient immune response inside the body cavity (Scharsack *et al.*, 2007; van der Veen and Kurtz, 2002). Nevertheless, in general, between 25% and 75% of the coracidia fail to reach the copepod's body cavity and the *S. solidus* prevalence in the stickleback varies from 25% to 50% in laboratory infections in our study population in Northern Germany (Hammerschmidt and Kurtz, 2005a; Jäger and Schjørring, 2006; unpublished observation). This also varies among populations and years (Arme and Owen, 1967; unpublished observation).

5.3.2.1. Copepod

In the copepod, after ingestion, the oncosphere must be liberated from the coracidium shell (Fig. 5.6) and pass through the pharynx and oesophagus. Some of the oncospheres are destroyed during this passage and as a result, never reach the copepod stomach or mid-gut. This is where the survivors penetrate the epithelium (Fig. 5.5A) using their penetration glands (Dubinina, 1966). The peritrophic membrane, a thin layer of protein and chitin lining the gut of arthropods, might shield against *S. solidus* oncospheres (Yoshikoshi and Ko, 1988). It has been shown to serve as an innate defence barrier against viruses and other microbial pathogens (Lehane, 1997). Previous studies reported the presence of brown spots along the gut surface (Fig. 5.2B), which could potentially contain encapsulated parasites (van der Veen and Kurtz, 2002). *S. solidus* sibships (genotypes) differ strongly in their ability to infect copepods (Hammerschmidt and Kurtz, 2005a; Wedekind and Rüetschi, 2000). Likewise, copepod sibships differ in susceptibility to tapeworm infection (Lena Sivars-Becker, personal communication). Although not tested directly, this gives potential for genotype-specific interactions between host and parasite, that is, parasites display infectivity specific to particular host genotypes, and hosts demonstrate resistance specific to particular parasite genotypes (see Kurtz, 2007). Such genetic specificity is considered important when hosts and parasites co-evolve (Carius *et al.*, 2001). It is likely that the result of the parasite overcoming several specific and non-specific host defence barriers, will lead to an overall specific outcome (Schmid-Hempel and Ebert, 2003). In the copepod, non-specific

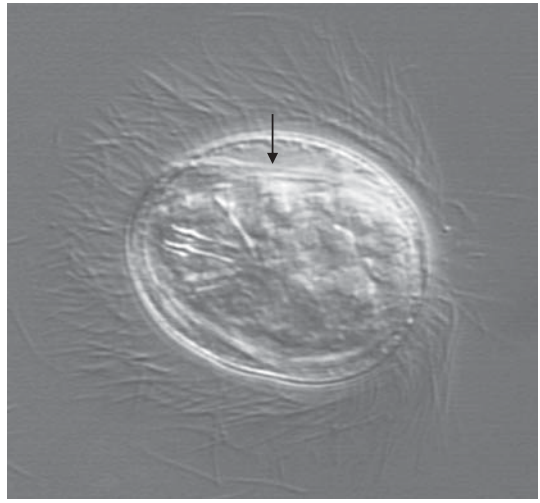

FIGURE 5.6 The *S. solidus* oncosphere (arrow) is visible within its coracidium shell.

mechanisms are most probably involved before *S. solidus* penetrates the gut epithelium, such as the likelihood of the oncosphere to get liberated from its coracidium shell and surviving the pharynx and oesophagus with its strong muscular contractions (Dubinina, 1966). Another unspecific factor might be the time needed to reach the copepod's body cavity without being digested. For instance, experimental infection rates were found to be considerably higher in briefly (2 days) starved animals, even when ingestion rates did not differ. This might be based on the fact that food items in the digestive system hinder or potentially damage the parasite (Hammerschmidt, unpublished observation). Once the parasite reaches the gut epithelium and starts penetration, unspecific immune defences of the copepod will presumably be triggered as a first-line defence. Also more specific interactions could take place, caused by variation in parasite or host properties, for example, parasite glands with the secretions to dissolve the chitineous layer, which in turn could be of differing thickness (as discussed in van der Veen and Kurtz, 2002), and therefore represent a potential explanation for the differences in susceptibilities of different copepod age classes or phenotypic qualities.

Apart from that, there is also evidence to suggest that tapeworms vary in properties recognised by the copepod's immune system, as line-specific immune memory has been found in copepods when comparing the prevalence of infection after a prior exposure to the same *S. solidus* genotypes (Kurtz and Franz, 2003). Specificity here refers to an induced and specific immune reaction, whereas genotype-specific interactions refer to genetically fixed specificities. The mechanism underlying specific immune memory could be that lectins bind to specific carbohydrates (Kurtz and Franz, 2003), as parasite sibships have been shown to possess variable carbohydrate surface composition (Hammerschmidt and Kurtz, 2005b). Alternatively, specific memory might be based on somatically diversified immune receptors, such as Down's syndrome cell adhesion molecule (DSCAM), which has been suggested to provide an alternative form of adaptive immune receptors in invertebrates (Dong *et al.*, 2006; Kurtz and Armitage 2006; Watson *et al.*, 2005). However, the topic of immunological specificity and memory in invertebrates has been reviewed elsewhere and is thus not the focus of the current review (Kurtz, 2004, 2005; Little *et al.*, 2005; Schulenburg *et al.*, 2007).

5.3.2.2. Stickleback

As in the copepod, after having entered the host via the oral route, the parasite has to survive unspecific barriers initially before experiencing specific barriers. It has to pass quickly through the acidic environment of the stomach lumen, seemingly protected by a thin outer membrane, which is lost in the gut (Hammerschmidt and Kurtz, 2007). When reaching the gut lumen, procercoids most likely attach with their microtriches

(used for nutrient uptake and movement; see Mehlhorn, 2001 for details), lyse the epithelial intestine layer, migrate through body contractions into the lamina propria, before passing through its large intercellular spaces into the stickleback's body cavity (Hammerschmidt and Kurtz, 2007; Fig. 5.5B). This process has been described for other helminths, such as *Taenia* and *Echinococcus* oncospheres (Barker, 1970; Heath, 1971). Also here, site-specific immune interactions between the invading procercoid and the stickleback intestine and intestinal epithelium should take place as in teleost fish several types of leucocytes (macrophages, lymphocytes, mast cells and granulocytes) are present in the gut (Georgopoulou and Vernier, 1986; McMillan and Secombes, 1997). The oxidative burst reaction might be particularly strong in the gut, where harm to self-tissue might be less severe than in other host tissues (Read and Skorping, 1995). Gut immune reactions might be so severe that avoiding them has even been discussed as one explanation for the tissue migration of many parasitic helminth (Mulcahy *et al.*, 2005; Read and Skorping, 1995). In spite of its potential relevance, gut immunity against *S. solidus* has not been studied in detail in the stickleback host.

5.3.3. Immunity in the body cavity

Only 3% of copepods, initially infected with multiple parasites (day 1 PE), became completely parasite-free later during infection (van der Veen and Kurtz, 2002). Furthermore, encapsulation, an invertebrate immune reaction particularly effective against objects larger than 10 μm (Grimstone *et al.*, 1967), of *S. solidus* procercoids, has occasionally been observed along the gut surface but only very rarely elsewhere inside the copepod haemocoel (van der Veen and Kurtz, 2002; Hammerschmidt and Kurtz, unpublished observations; Fig. 5.2B). Similar observations have been reported for the stickleback. Only during early infection (prior to day 17 PE), prevalence marginally decreased and dead parasites were occasionally found in the body cavity (Scharsack *et al.*, 2007). This indicates that for *S. solidus*, the risk to be cleared by the host immune response is highest during early infection, but negligible thereafter (Scharsack *et al.*, 2007). The establishment probability of tapeworm sibships (used as a proxy for genetic lines) in the stickleback was negatively correlated to the occurrence of certain types of carbohydrates on the plerocercoid's surface, but positively with the innate immune response (respiratory burst reaction). This suggests that parasite recognition might be mediated by the tapeworms' surface carbohydrates (Hammerschmidt and Kurtz, 2005b). The stickleback immune system clearly recognises the invading parasite as (i) there is variation in innate immune reaction correlated with surface characteristics (Hammerschmidt and Kurtz, 2005b) and (ii) immune cells of infected sticklebacks are mobilised at day 7 PE as monocytes (precursors of

macrophages and granulocytes) proliferate (Scharsack *et al.*, 2007). Nevertheless, in infected compared to control fish, the respiratory burst activity (the key function of cell-mediated innate immunity against invading macroparasites) is not elevated during the early *S. solidus* infection (Scharsack *et al.*, 2007; Hammerschmidt and Kurtz, unpublished data). This is surprising, given the relevance of the early stages of infection for parasite elimination as described above (Scharsack *et al.*, 2007).

5.4. PHASE II: RESOURCE ACQUISITION AND IMMUNE EVASION

We define this phase to start after the parasite has reached the body cavity of either intermediate host and to end when the parasite is infective for the next host in the cycle.

In the copepod, the development time from the oncosphere to the invasive procercoid depends on (i) temperature, (ii) copepod species, (iii) copepod size and (iv) number of procercoids present in the copepod (Dubinina, 1966; Michaud *et al.*, 2006; Wedekind and Jakobsen, 1998; Wedekind *et al.*, 2000). Under laboratory conditions, that is, with singly infected *M. albidus* copepods fed *ad libitum* and kept at 18 °C, first infections of sticklebacks could be achieved with 11-day-old procercoids (Hammerschmidt *et al.*, unpublished data). Morphological premises that have to be fulfilled in order to be infective to the stickleback are the formation of the cercomer, calcareous corpuscles and the development of the penetration glands (Dubinina, 1966; Orr and Hopkins, 1969).

In the stickleback, plerocercoids are known to reach infectivity for the definite bird host and are able to produce fertile eggs *in vitro*, when reaching 50 mg in weight (Tierney and Crompton, 1992). The main factors determining the time needed to reach the size for maturity are (i) temperature, (ii) fish size, (iii) number of plerocercoids per fish, (iv) size/age of invading procercoid (Barber, 2005; Dubinina, 1966; Hammerschmidt *et al.*, unpublished data; Orr and Hopkins, 1969; Sinha and Hopkins, 1967). In single infections of young-of-the-year sticklebacks, this takes approximately 45 days at 18 °C (Scharsack *et al.*, 2007). What information is available on this phase of infection?

5.4.1. Copepod

While copepods seem unable to eliminate *S. solidus* procercoids after establishment in the body cavity (van der Veen and Kurtz, 2002), they can limit the growth of the tapeworms, since immune induction by prior exposure of the copepod to sibling worms resulted in a reduced size (Kurtz and Franz, 2003). Procercoids might differ in their ability to

camouflage, and thus reduce recognition and attack by the immune system. Two carbohydrates that have been reported to be of importance in interactions between insect vectors and their parasites, GalNAc and D-galactose (Burton *et al.*, 1999; Knowles *et al.*, 1991) were detected on the procercoid surface with lectin labelling (Hammerschmidt and Kurtz, 2005b; Jacobson and Doyle, 1996). However, in this study no relationship was found between parasite performance (infectivity and size) and these surface characteristics in the copepod host (Hammerschmidt and Kurtz, 2005b). Alternatively, surface components may protect the parasite from enzymatic digestion and/or immune defence in the next (stickleback) host, as has been found in other systems (Ingold *et al.*, 2000; Obregón-Henao *et al.*, 2003; Sandeman and Williams 1984). This is more likely here as (i) the procercoid surface was analysed at 12 days PE in this study and at a time where procercoids are able to infect the next host, and (ii) the transformation from the oncosphere to the fully developed procercoid involves complex morphological changes, including the tapeworm's outer body layers (Dubinina, 1966). This does not exclude the role of surface sugars early on in copepod infection, but merely implies that for a clearer understanding, more information on the surface properties of *S. solidus* procercoids during the whole course of infection is needed.

Other than hiding from the immune systems, many parasites take a more active role and excrete substances to suppress or modify host immunity (reviewed in Damian, 1997). We do not have any direct evidence for such parasite manipulation, but indications suggest that:

(i) During infection, already at day 7 PE, the number of orange carotenoid droplets increases (Hammerschmidt, 2006; Fig. 5.2D). Carotenoids are important free-radical scavengers, which protect host tissue from oxidative damage during immune defence. The immune response often involves free-radical production (Bendich, 1989, 1993; von Schantz *et al.*, 1999). During an on-going infection, the carotenoid storage should therefore decrease, which is contrary to the findings. This suggests that the immune system is repressed and carotenoids are therefore not used (Hammerschmidt, 2006). Alternatively, tapeworms may directly manipulate carotenoid storage to increase the visibility of infected copepods for the stickleback host, which uses optical cues for prey detection.

(ii) Parasites seem able to sense the number of con-specific competitors, as they adjust their growth accordingly (Michaud *et al.*, 2006). This is unlikely to be due to resource restriction, as the total parasite volume of multiple infections exceeds that of single ones (Michaud *et al.*, 2006). If parasites do not purely adjust their growth to the available resources, there should be (chemical) cues released from procercoids, which may also act as host manipulators.

(iii) Activity of infected copepods significantly decreases at 5 days PE, whereas simultaneously, recovery time from a simulated predator attack increases (Hammerschmidt, 2006; Hammerschmidt *et al*., unpublished data). This change in copepod behaviour is unlikely to be caused by energy-depletion as a side effect of the infection, as no effect of parasite infection on muscles or storage lipids has been detected even at a later stage of infection (9 days PE) (Franz and Kurtz, 2002). Moreover, the timing of the behavioural change precisely coincides with infectivity for the next host (Parker *et al*., 2009; Hammerschmidt *et al*., unpublished data; more details below).

No later than 5 days PE the procercoid seems able to manipulate host behaviour and immune defence (Hammerschmidt, 2006; Hammerschmidt *et al*., personal communication), thus establishing full control over its host. It may take time for the procercoid to reach a sufficient size for secreting manipulative substances. At day 2 PE, the larva starts growing exponentially until it reaches an asymptotic size at around day 30 PE (Dubinina, 1966). Growth of the parasite is supposed to be optimised based on a virulence trade-off between growth and reduced transmission due to host death (Michaud *et al*., 2006; Parker *et al*., 2003b).

5.4.2. Stickleback

5.4.2.1. Parasite growth

After establishment, plerocercoids of *S. solidus* grow exponentially and double their weight every 4–5 days (Orr and Hopkins, 1969; Fig. 5.1). Their growth rate is highest between day 7 and 17 PE, when the parasite increases its size by approximately 17 fold (at 18 °C in single infections and under optimal laboratory conditions). This could be a strategy to outgrow the size at which it is still vulnerable to elimination by the host. Up until 17 days PE, dead parasites have been found in the stickleback's body cavity (Scharsack *et al*., 2007; Kurtz, unpublished observation). Within less than 6 weeks, parasites increase their weight by approximately 4000 fold (Scharsack *et al*., 2007). Apart from external factors (see above), plerocercoid growth is influenced by performance in the copepod. A recent study found that the age of the procercoid upon transfer to the stickleback not only influenced infectivity in the stickleback, but also its growth until reaching the infective size for the final host (50 mg at day 45 PE) (Hammerschmidt *et al*., unpublished data). Here, procercoids have an optimal transfer time (12.6–15.2 days PE) from the copepod to the stickleback host, which leads to maximum fitness in the final host in terms of production of viable offspring (Hammerschmidt *et al*., unpublished data). Additional factors restricting parasite growth are host immunogenetics. Sticklebacks with an optimal number of major histocompatibility complex

(MHC) genes were found to harbour smaller plerocercoids (Kurtz *et al.*, 2004), and carbohydrates on the parasite surface (Hammerschmidt and Kurtz, 2005b). We found that tapeworms possessing more vertebrate-like carbohydrates, such as GlcNAc and sialic acid residues, were inferior at infecting, but superior in growing in the stickleback host (Hammerschmidt and Kurtz, 2005b). One potential reason might be that tapeworm surfaces resembling invertebrate or vertebrate surfaces cope best with either the innate or the adaptive component of the stickleback immune system, with the former counteracting the establishment of an infection, while the latter seems to constrain the growth of the worm (Hammerschmidt and Kurtz, 2005a).

5.4.2.2. Host immunity

A characteristic innate immune response of fish against helminth parasites is the mobilisation and activation of granulocytes (Hoole and Arme, 1983; Nie and Hoole, 2000). These produce oxygen intermediates such as nitric oxide (NO) or reactive oxygen species (ROS) to destroy invading pathogens with a so-called oxidative burst (Secombes and Chappell, 1996; Whyte *et al.*, 1990). In addition to this cellular innate defence, the adaptive immune system produces specific antibodies against parasite antigens (Roberts *et al.*, 2005; Wiegertjes *et al.*, 2005). Activation of the specific immune system is typically measured by assessing the proliferation of lymphocytes (Le Morvan-Rocher *et al.*, 1995; Nie *et al.*, 1996).

The oxidative burst reaction is considered to be of great importance against small tapeworm stages, as it is not clear whether large helminth parasites can also be killed by cellular immune responses *in vivo* (Secombes and Chappell, 1996). Indirect evidence, from transplant studies, suggests that the fish immune system is, in principle, able to destroy *S. solidus* by cellular innate responses (Bråten, 1966); early *S. solidus* plerocercoids died between 2 to 10 days after transplantation from their original stickleback host to other fish species. However, they survived when transplanted into another stickleback. In a recent study that investigated immune defence during experimental infections, sticklebacks infected with *S. solidus* did not up-regulate the oxidative burst reaction early on when plerocercoids were still small (Scharsack *et al.*, 2007). Taken together, this indicates that *S. solidus* plerocercoids either suppress or avoid the immune system of the stickleback, but are unprotected against the immune defences of other fish species. Parasites may hide from the host's immune system (Aeschlimann *et al.*, 2000), for example, by modifying their surface carbohydrate composition to that of their vertebrate host (as discussed above and in Hammerschmidt and Kurtz, 2005b). Findings from another study suggest an active immune suppression: leukocytes of infected sticklebacks did not show an oxidative burst reaction when stimulated with *S. solidus* extract *in vitro* (in contrast to naïve sticklebacks) but do so against a more general stimulus such as poke weed mitogen

(PWM; so that a general anergy of these cells can be excluded) (Scharsack *et al.*, 2004). Moreover, leucocytes from *S. solidus*-infected fish did not respond to stimulation with *S. solidus* antigens *in vitro*, whereas sticklebacks that had successfully cleared an infection (exposed but non-infected) showed a higher ROS production than the control fish (Scharsack *et al.*, 2004). Additional evidence for active immunosuppression comes from a study that measured *S. solidus* growth in sequential infections, where sticklebacks were exposed to a second parasite 1 week after the first exposure. The second parasite grew larger even though it was 1 week younger (Jäger and Schjørring, 2006). One potential explanation for this phenomenon could be that there are higher costs associated with being the first parasite and needing to suppress the immune system. Taken together, these results give compelling indirect evidence for active immunosuppression in addition to surface mimicry (Hammerschmidt and Kurtz, 2005b).

5.5. PHASE III: HOST SWITCH AND MANIPULATION

Theoretical models predict that the basis for the evolution of complex parasitic life cycles is the fitness advantage through the incorporation of additional hosts. Parasites should gain in terms of reaching a bigger final size or benefit from an increased transmission success between hosts (Choisy *et al.*, 2003; Iwasa and Wada, 2006; Parker *et al.*, 2003a). One way for the parasite to enhance transmission probability to the next host even further is to make it a more attractive prey for the following host in the cycle. Transmission-enhancing behavioural alterations of intermediate hosts during parasite infection have been of interest to parasitologists for several decades (Bethel and Holmes, 1974; reviewed by Moore, 2002; Thomas *et al.*, 2005), and are typical examples for the 'extended phenotype' concept (Dawkins, 1982), where genes of one organism (here the parasite) have phenotypic effects in another organism (the host). However, only a few studies have used experimental infections and have been able to show conclusively that changes in host behaviour are adaptive for the parasite rather than a side effect of infection (Berdoy *et al.*, 2000; see Poulin 1995 for review). In the *S. solidus* system, changes in the behaviour and phenotype of infected intermediate hosts have been studied intensively.

5.5.1. Copepod

In its first intermediate host, an infection with *S. solidus* was found to lead to decreased escape ability, an increased general activity and changes in micro-habitat choice (Jakobsen and Wedekind, 1998; Urdal *et al.*, 1995). These changes in behaviour would lead to a higher parasite transmission rate to the next host in nature as three-spined sticklebacks preferred

infected copepods over non-infected ones or other food sources, such as daphnids (Urdal *et al*., 1995; Wedekind and Milinski, 1996). It is unclear as to what exactly makes infected copepods a preferred prey compared to non-infected ones. In general, the more active copepods were attacked more often, but on top of that, infected copepods were still more often attacked per unit activity than non-infected ones (Wedekind and Milinski, 1996). One reason could be a change in the type of movement (e.g., to appear as weaker swimmers) (Wedekind and Milinski, 1996) or other signals that sticklebacks prefer (e.g., an enhanced orange appearance due to an increased storage of carotenoids) (Hammerschmidt, 2006; Fig. 5.2D).

Previous studies, which investigated whether this change in copepod behaviour is adaptive or a side effect of infection, came to different conclusions: one study found the behavioural changes to be hunger induced, indicating a side effect (Jakobsen and Wedekind, 1998), whereas another study suggested it to be a direct manipulation rather than a consequence of energy limitation or resource allocation (Franz and Kurtz, 2002). The latter finding is supported by a study that monitored growth patterns of *S. solidus* procercoids in single, double and triple infections (Michaud *et al*., 2006). Here, the total volume of procercoids in multiple infections was larger than that for single infections (even though the individual procercoids were proportionally smaller), which shows that more resources are available than used by a single infecting procercoid.

One criterion for manipulation to be adaptive is that it must increase the fitness of the parasite (Poulin, 1995). Thus, the change in behaviour should only occur after the parasite becomes infective for the next host but more specifically around the optimal transfer time, as predicted by recent theoretical models (Parker *et al*., 2003b; Parker *et al*., 2009; Hammerschmidt *et al*., unpublished data). This was indeed shown for procercoids of *S. solidus* in their copepod hosts. When procercoids were transmitted at the optimal transfer time, parasite performance in the second intermediate and final host, as well as fitness in the next generation (viable offspring), were all maximised (Hammerschmidt *et al*., unpublished data). Around this time, a change in behaviour from predation suppression to predation enhancement occurred, as predicted by Parker *et al*. (2009). *S. solidus* is one of a few systems, where both predation suppression (here a decrease in activity, and increase in time to recover from a simulated attack, before reaching the infective stage for the next host) and predation enhancement at the optimal transfer time could be demonstrated (Hammerschmidt *et al*., unpublished data; Parker *et al*., 2009). These findings have general implications for how and when to check for parasite manipulation: predation enhancement should only start when the optimal transfer time is reached, and not already at the time, when the parasite is infective for the next host. This discrepancy could potentially explain why some studies failed to detect changes in host behaviour or the next host did not prefer infected intermediate hosts.

5.5.2. Stickleback

As in the copepod host, an infection with *S. solidus* is known to change the behaviour and the phenotype of the three-spined stickleback. Among others, changes in host appearance (Lobue and Bell, 1993), microhabitat choice (Jakobsen *et al.*, 1988; Lobue and Bell, 1993), activity (Giles, 1983; Milinski, 1985) and evasive behaviour (Milinski, 1990) have been reported. Infected sticklebacks also change their response to predators as they recover more quickly from a simulated attack (Giles, 1983) and forage closer to potential fish predators (Milinski, 1985). One study, which compared the anti-predator behaviour of sticklebacks harbouring (i) none, (ii) for the bird uninfective, or (iii) infective *S. solidus* plerocercoids (Tierney *et al.*, 1993) obtained comparable results to the study monitoring copepod anti-predator behaviour during the course of an *S. solidus* infection (see above; Hammerschmidt *et al.*, unpublished data). Sticklebacks with infective plerocercoids recovered quickest from a simulated predator attack (Tierney *et al.*, 1993), indicating enhanced predation (Parker *et al.*, 2009). This switch between predation suppression and enhancement strongly indicates parasite manipulation, especially as these changes occurred in saturated sticklebacks, that is, hunger-induced behavioural changes as proposed by Giles (1987) can most probably be excluded (Tierney *et al.*, 1993).

What mechanism could be responsible for these changes in host behaviour? Scharsack *et al.* (2007) found that the immune system, especially the oxidative burst reaction, was not up-regulated during the infection until the plerocercoid reached 50 mg, the threshold weight for the parasite to infect and produce fertile eggs in the final host successfully (Tierny and Crompton, 1992). After that, infected fish showed significantly higher oxidative burst reactions than the control fish (Scharsack *et al.*, 2007). Paradoxically, that late during infection, the observed up-regulation of innate immunity is unlikely to help the host, as the parasite has reached a size too large for elimination, while immunity itself bears immunopathological costs (Hammerschmidt and Kurtz, 2005a; Kurtz *et al.*, 2006; Lochmiller and Deerenberg, 2000; Scharsack *et al.*, 2007). At the same time, infected sticklebacks show a reduced predator avoidance behaviour, which is most likely to be caused by an increase in monoamine neurotransmitter concentrations (Øverli *et al.*, 2001). It is currently unknown whether the increase in neurotransmitter results from active parasite manipulation of the host neuroendocrine system, or from chronic stress as a side effect of parasite infection and the associated immune responses (Øverli *et al.*, 2001). Distinguishing between these alternatives is difficult due to the tight and complex interactions between the immune and nervous system (Adamo, 2002; Thomas *et al.*, 2005). Studies on invertebrates suggest that parasite secretions activate components of the host's immune system, and thereby manipulate its nervous system (Adamo, 2002).

This could also be the case in sticklebacks, as for vertebrates it is also known that immune-mediated behavioural changes occur during acute infections (Vollmer-Conna, 2001). Mediated through the up-regulation of the respiratory burst activity at the optimal moment, that is, when ready for host switch, *S. solidus* could trigger transmission-enhancing neuronal and behavioural modifications (Scharsack *et al.*, 2007).

5.6. LINK BETWEEN HOSTS IN COMPLEX LIFE CYCLES

The evolution of complex life cycles has received much theoretical interest (Ball *et al.*, 2008; Choisy *et al.*, 2003; Gandon, 2004; Iwasa and Wada, 2006; Parker *et al.*, 2003a, 2009). Parker *et al.* (2003a), for example, developed a useful concept for the understanding of the evolution of complex parasite life cycles. In principle, parasite fitness increases when a new host is added to the cycle as typically host (and thus also parasite) mortality decreases and transmission probability between hosts, parasite size and fecundity increases (Parker *et al.*, 2003a). This concept is based on growth optimisation of the parasite in its host, as mediated mainly by resource acquisition from the host. Inevitably, the model in its current form largely ignores the potential immunity of the hosts against the parasite as an effect contributing to variation in parasite growth and transmission probability and timing. However, recent empirical studies on parasites with complex life cycles (Hammerschmidt and Kurtz, 2005a; Mackinnon and Read, 2004) in combination with results from serial passage experiments (Ebert 1998), clearly show that adaptation and performance in the intermediate hosts have an impact on parasite performance and virulence in the next (and/or final) host. In addition, the present review suggests that immunological interactions between host(s) and parasite(s) are relevant factors influencing not only parasite establishment and growth, but potentially also behavioural manipulation of the hosts. Clearly, the latter aspect needs further investigation, including its mechanistic underpinnings. Nevertheless, theoretical models on parasite complex life cycles should also start to include host immunity.

5.7. CONCLUDING REMARKS

The different phases during an *S. solidus* infection seem to be similar in both hosts. At the start of the infection, the outcome of an exposure resembles a yes–no decision, and depends on the properties of the host, the parasite and on the interaction between the two, but also on several unspecific (external) processes. Shortly after successful parasite establishment, *S. solidus* 'extends its phenotype' (Dawkins, 1982), starts to dominate the

interaction and takes control over its hosts, finally resulting in the manipulation of host behaviour, leading to enhanced transmission to the next host. In this context, most studies have so far concentrated mainly on the ultimate factors, that is, evolutionary causes of the behavioural change of hosts (Parker *et al.*, 2009; Poulin, 1994, 1995). By contrast, the present review emphasises a promising extension of the extended phenotype concept, such that its proximate, physiological underpinnings are included. When these were addressed in previous studies, the focus was mainly on mechanisms that directly serve to manipulate the host, such as host neuromodulators (Adamo, 2002; Thomas *et al.*, 2005). Here we suggest that parasite-induced behavioural changes of the host may also be caused by interactions of the parasite with the host immune system, which are influenced by the antigenic surface of the parasite. To understand host–parasite interactions fully, more information on the molecular crosstalk between the host immune, hormone and nervous system is needed. Technical advancements, such as new '-omics' tools that have now become available for non-classical model organisms, will facilitate further in-depth studies of host–parasite interactions in the near future (Biron *et al.*, 2005; Lefèvre *et al.*, 2006). The extended phenotype concept can then be expanded and parasitised hosts can truly be seen as 'deeply modified organisms.' (Thomas *et al.*, 2005).

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CHAPTER 6

Tracking Transmission of the Zoonosis *Toxoplasma gondii*

Judith E. Smith

Contents

Abstract

Toxoplasma gondii is a highly successful parasite that infects many host species and has colonised a wide range of habitats. Review of the parasite's life cycle demonstrates that it has become adapted to exploit multiple routes of transmission through a sexual cycle in the definitive host and asexually, through carnivory, and by vertical transmission. These alternative routes may operate synergistically to enhance transmission, but they might also provide a vehicle for selection leading to partitioning of strains in the

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environment. Genetic analysis has shown that parasite population structure varies globally. In South America, there is high strain diversity while in North America, Europe and Africa three clonal strain types predominate. This may imply a shift from sexual to asexual transmission. Mapping of the parasite genome has provided a wealth of markers for strain characterisation. Close genotyping of isolates gives evidence of multiple infection and recombination in natural populations and reveals differences in both the distribution and the phenotype of strains. More intensive epidemiological studies are now required to unravel the networks of transmission operating within defined habitats.

6.1. TOXOPLASMA: THE SUPREME GENERALIST

Toxoplasma is regarded as the most common protozoan parasite having colonised many host species over a vast range of habitats (Lindsay and Dubey, 2007; Tenter *et al*., 2000). Although this zoonotic parasite was initially discovered in a wild North African rodent (Nicolle and Manceaux, 1908), studies of its epidemiology have inevitably focused on disease risk in humans and in domesticated species. In humans, infection is common and symptoms are normally mild and transient, however, the parasite can prove fatal to immunosuppressed individuals and, if acquired during pregnancy, can cause abortion or serious congenital disease (Jones *et al*., 2002; Remington *et al*., 2006). The prevalence of Toxoplasma infection in the human population varies globally (Tenter *et al*., 2000). Recent studies show an overall prevalence of 10% in the United States (Jones *et al*., 2007) and 24% in Northern Greece (Diza *et al*., 2005), but infection rates can be much higher elsewhere in the world reaching 70% in Indonesia (Terazawa *et al*., 2003) and over 80% in indigenous Brazilian tribes (Sobral *et al*., 2005). Congenital toxoplasmosis is estimated to affect one in 12,000 births in the United States (Jara *et al*., 2001) rising to one in 3,000 in Brazil (Neto *et al*., 2000) and one in 1,000 Poland (Paul *et al*., 2001).

Toxoplasma infection is very common in domesticated species (Dubey and Beattie, 1988; Lindsay and Dubey, 2007; Tenter *et al*., 2000). The parasite is particularly important in sheep, where it is a significant cause of abortion (Buxton *et al*., 2007) but infection is well documented in pigs (Dubey, 1986) and chickens (Dubey, 2008). The parasite has also been reported from goats (Bisson *et al*., 2000), llamas (Chavez-Velasqueza *et al*., 2005) and can infect cattle, horses and dogs, although these animals appear quite resistant to disease (Lindsay and Dubey, 2007). The ability of the parasite to infect so many species gives ample opportunity for the operation of peridomestic transmission cycles. The domestic cat, as a definitive host for the parasite, is positioned to play a central role here (Dubey, 2007; Hutchinson *et al*., 1971).

The parasite is not, however, restricted to the human environment but is detected in a wide range of habitats. In the African savannah, evidence of infection is found in kudu, bushbuck, giraffe, elephant, ostrich and lion (Hove and Mukaratirwa, 2005), in the arctic it is found in arctic fox, barnacle geese, walrus and polar bear (Prestrud *et al.*, 2007; Rah *et al.*, 2005) and in the ocean it is found in dolphins (Dubey *et al.*, 2008a), sea otters (Conrad *et al.*, 2005) and seals (Lambourn *et al.*, 2001).

Although many protozoan parasites have zoonotic transmission, *Toxoplasma* might be regarded as the supreme generalist with largest number of intermediate hosts. The parasite does not recognise phylogenetic barriers, being found in divergent host species with vast differences in physiology and life history, neither is it restricted by habitat. It is interesting to question how this parasite achieves such a broad distribution among host taxa and which adaptations have led to its remarkable success.

6.2. ADAPTATION FOR TRANSMISSION

The immense host range of *Toxoplasma gondii* and the diverse habitats it has colonised parasite suggest that the parasite must have very flexible strategies for transmission. Consideration of the life cycle gives some insight into this. Like other members of the phylum apicomplexa, *Toxoplasma* has both a sexual and an asexual cycle. However, unlike parasites such as malaria, Babesia and Eimeria, this sexual cycle is an optional rather than a fixed component and there are opportunities for direct transmission through the asexual cycle (Smith and Rebuck, 2000).

6.2.1. Life cycle and transmission

The sexual stage of the life cycle takes place only in the felid definitive host. Both domestic and wild species, such as bobcats and cougars, have been shown to transmit the infective oocysts stage (Aramini *et al.*, 1998; Dubey, 2007; Millar *et al.*, 1972). Following oral infection, parasites invade the epithelial cells of the intestine to produce a series of morphologically distinct life stages leading to the production of gametocytes (Speer and Dubey, 2004; Speer *et al.*, 1997). Micro- and macro-gametes are released and fertilisation occurs resulting in the formation of oocysts that are passed out in the faeces. On release into the environment, oocysts mature to form a double envelope with an outer oocyst wall containing eight haploid sporozoites packaged within two sporocysts. Oocysts are normally produced for a brief period during primary infection but a single animal can excrete millions of oocysts over this time (Dubey, 2006). The oocyst is an important stage in the transmission of all cyst-forming

coccidia and is highly adapted to survive in the environment. Toxoplasma oocysts can withstand periods of freezing at –20 °C and temperatures as high as 50 °C. They have been shown to survive for over 2 years at 4 °C (Dubey, 1998a; Frenkel *et al*., 1975). Oocysts are highly infective to intermediate hosts, a single oocyst is sufficient to establish infection in mice but high doses are required to infect cats (Dubey, 2006, 2007).

The asexual cycle takes place in a wide range of intermediate hosts. Following ingestion, the oocyst releases sporozoites, which invade the gut epithelia and transform into tachyzoites (Dubey *et al*., 1997a; Tilley *et al*., 1997). This stage grows rapidly and spreads from the gut colonising many tissues. The burden of infection is high in this early acute phase and elicits a strong immune response that suppresses tachyzoite growth. From the earliest time point, tachyzoites have the potential to differentiate into bradyzoites. These slow-growing stages form thick-walled tissue cysts, which persist for long periods in the host (Dubey *et al*., 1997a; Ferguson *et al*., 1989). Stage conversion between tachyzoites and bradyzoites is reversible with the outcome that a chronic 'steady state' infection is maintained by periodic release of tachyzoites and reconversion to form new tissue cysts (Soete *et al*., 1993; Weiss and Kim, 2007). When a host is immunosuppressed this balance is lost and recrudescence of tachyzoites can lead to disease reactivation (Luft and Remington, 1992; Odaert *et al*., 1996). There are two opportunities for the transmission of asexual stages, through the food chain and via vertical transmission. Bradyzoites encysted in the tissues of an infected animal can transmit the infection directly to a new host. This part of the life cycle is important for the infection of cats, which are highly susceptible to bradyzoites (Dubey, 2001) as well as for transmission to intermediate hosts (Dubey, 2006). Both bradyzoites and tachyzoites are able to transmit infection orally but the infective dose is much higher for tachyzoites (Dubey, 1998b). Bradyzoites can survive in the carcass for a considerable time but studies on meat for human consumption show that they are unable to survive freezing at –20 °C or cooking at 66 °C (Dubey *et al*., 1990; Kotula *et al*., 1991). The second mechanism of asexual transmission is through congenital infection. It is well established that the tachyzoite has the ability to cross the placenta and infect the developing foetus (Remington *et al*., 2006). There is evidence that in some species this might lead to serial vertical transmission, which would sustain the parasite in the population over generations (Beverley, 1959; Morley *et al*., 2005).

6.2.2. Multiple transmission routes

Review of the life cycle of Toxoplasma reveals that there are three major potential routes of transmission, via the cat, through carnivory, and by vertical transmission. These transmission routes have been validated

by experimental studies in mice and sometimes in other species. Experimental studies may not, however, be analogous to transmission in nature as models are restricted, parasites have been maintained in culture for a long time and doses used are sometimes high. It is in fact very difficult to evaluate the relative importance of different transmission routes in the field and most evidence we have is indirect.

The ingestion of oocysts derived from the cat is inevitably cited as main mechanism of transmission. There is a proven ability for oocysts to infect mice and some domesticated species in experimental studies (Dubey *et al*., 1996, 1997a; McColgan *et al*., 1988). Evidence in support of oocyst-mediated infection in nature comes from several sources. Methods for detecting Toxoplasma oocysts in environmental samples are poorly developed, making it very difficult to estimate the density and distribution of oocysts in soil, water or forage (Dumètre and Dardé, 2003). Thus, although associations have been made between outbreaks of toxoplasmosis and exposure to oocysts, there is only one instance when contemporary isolation of oocysts was made from soil (Goutinho *et al*., 1982). Other reports infer the source of the epidemic as soil or water (Benenson *et al*., 1982). One of the best-documented studies is of a 6-month epidemic in Vancouver Island, which was linked to use of water from a local reservoir (Bowie *et al*., 1997). Oocysts were isolated from cougar faeces collected from the watershed area, but were not found in reservoir itself (Aramini *et al*., 1998). Studies of toxoplasmosis in pigs note that there are large differences in the prevalence of the parasite between herds that reflect different environmental and management regimens (Dubey and Jones, 2008). Cats were counted as an important risk factor for infection and oocysts were detected in cat faeces, soil and water on a number of farms (Dubey *et al*., 1995a; Weigel *et al*., 1999). A low prevalence of toxoplasmosis has also been noted in feral pigs living on an island without cats (Dubey *et al*., 1997b). Oocysts are the most likely source of infection in herbivores such as sheep or antelope (Hove and Mukaratirwa, 2005; Plant *et al*., 1974) and have also been proposed for transmission in marine ecosystems (Conrad *et al*., 2005). In these studies, as with the examples cited with human and pig populations, it remains difficult to quantify the importance of this route in different ecological niches.

Ingestion of bradyzoites in meat is an important mechanism of transmission in carnivores. Toxoplasma is common in meat produced for human consumption especially sheep, pigs, chickens and game (Tenter *et al*., 2000). There are reports of outbreaks of toxoplasmosis associated with ingestion of undercooked meat. Examples include a cluster of congenital cases in Inuit women linked to ingestion of uncooked carribou meat (McDonald *et al*., 1990) or an outbreak of acute toxoplasmosis in Korea associated with ingestion of raw pork

(Choi *et al*., 1997). However, as with oocyst-induced infection, there are no cases where parasites have been directly isolated from the proposed source of infection. Population surveys have linked consumption of undercooked meat with seropositivity in human populations and with the risk of on seroconversion during pregnancy (Baril *et al*., 1999; Cook *et al*., 2000). Very little is known about the role of the tissue cyst in transmitting infection among wild animals. This route should be most important in carnivorous species that might acquire Toxoplasma from prey and thus have a high rate of infection. The many point prevalence studies of toxoplasmosis in wild animals report some of the highest seroprevalence rates among carnivores such as bears, cats and foxes (Dubey *et al*., 1995b, 2004, 2007a; Tenter *et al*., 2000) and some of the lowest rates among insectivorous species, which should have low levels of exposure to both oocysts and bradyzoites (Dubey, 1983; Lindsay and Dubey, 2007). However, care should be taken in interpreting these studies as the level of variation between studies is immense and comparisons need to be placed in the appropriate environmental context.

Transplacental transmission of Toxoplasma has been demonstrated to occur in multiple species and is well studied in humans, rodents and sheep (Buxton *et al*., 2007; Remington *et al*., 2006; Schaap *et al*., 2007). Studies have mainly focused on congenital disease and it was noted that this was chiefly associated with primary infection during pregnancy. However, in some host species it was suggested that serial vertical transmission may occur. This was first proposed by Beverley (1959) following infection through generations of mice then later confirmed in transmission studies in both rats and mice (Owen and Trees, 1998; Webster, 1994). These experimental studies demonstrated that the potential for vertical transmission in a laboratory context but could not evaluate whether it occurred in nature. Research focusing on congenital infection of sheep demonstrated a high rate of congenital transmission with over 40% live-born lambs infected (Duncanson *et al*., 2001; Williams *et al*., 2005). The outcome of pre-natal exposure is that the animals may become asymptomatic carriers. Further, pedigree-based analysis revealed non-random patterns of disease in the flock and showed that there were familial associations between abortion risk and infection (Morley *et al*., 2005). Transplacental transmission occurs in many animals infected with Toxoplasma, including pigs, deer, sea otters and dolphins, but tests for serial vertical transmission have not been completed (Dubey *et al*., 1990, 2008b; Miller *et al*., 2008a; Resendes *et al*., 2002). It is possible that vertical transmission may be host species or host genotype specific, however, it is also possible that transmission also depends on environmental conditions. In species such as sheep, high levels of reproductive and nutritional stress may exacerbate pregnancy immunosuppression and allow reactivation of latent infection and transmission.

6.2.3. Potential for selection

Toxoplasma is highly adapted to exploit multiple transmission routes but there are still many questions as to how these operate. They could act synergistically to maximise transmission but there could be tension, as prospects for transmission between different hosts in different environments vary. Strains might become adapted to favour a specific route of transmission or to exploit a specific tropic interaction. For example, a strain that favoured vertical transmission might be selected for reduced pathogenesis and high transplacental transfer. Such strains are likely to be cryptic and difficult to detect in the environment. Conversely, a strain adapted for food-borne transmission might produce large numbers of tissue cysts and impair the survival of its host. If we consider this at the level an ecosystem, transmission could be completely flexible with all strains circulating randomly through all species, or it could be structured. One way to help unravel these networks and identify routes is to develop genetic markers that have the potential to detect and compare the identity of isolates.

6.3. PARASITE POPULATION GENETICS

Toxoplasma gondii is a single species with widespread distribution but genetic studies have revealed structure within parasite population. Application of these findings will allow testing to determine whether there is partitioning of strains between host groups or with habitat and to test associations between genotype and phenotype.

6.3.1. Genetic and biological diversity

It is well established that there are biological differences among Toxoplasma isolates. The description of the parasite phenotype is developed for a subset of strains that were isolated and maintained by passage either in mice or *in vitro*. The most striking variation is seen in the virulence to mice (Howe *et al.*, 1996), which subdivides strains into acute virulent (mouse lethal) and 'avirulent' phenotypes. Avirulent strains cause chronic stable infection in mice but differences in the severity of the acute phase, the risk of encephalitis and the parasite burden are still found (Ferguson *et al.*, 1994; Suzuki *et al.*, 1989). These differences in the pattern of infection in mice reflect multiple variations in the parasite phenotype. *In vitro* studies have differences in the efficiency of growth (Appleford and Smith, 1997; Kaufman *et al.*, 1959), and in the rate of tachyzoite–bradyzoite interconversion (Soete *et al.*, 1994). Similarly studies in mouse models show differences in transepithelial migration and

dissemination of virulent and avirulent strains (Barragan and Sibley, 2002; Saeij *et al*., 2005) and variation in the induction of host immune responses that might have significant effects on disease outcome (Robben *et al*., 2004). Interestingly there are also indications that isolates vary capacity for oral transmission between intermediate hosts and that this may be related to the efficiency of cyst production (Fux *et al*., 2007) while other strains vary in their infectivity to cats (Dubey, 1995). These data are supplemented by observations of phenotype in natural hosts at the time of isolation where disease association is noted (Dardé *et al*., 2007; Howe and Sibley, 1995). There are very big differences in data from the field and mouse studies and many isolates isolated from clinical infection appear avirulent in mice so care is needed when defining the phenotype.

Numerous molecular markers have been developed to establish the extent of genetic diversity within *Toxoplasma gondii*. Initially phenotypic markers were used to compare isolates. There are considerable differences in the antigenic structure of parasite strains (Appleford and Smith, 2000; Ware and Kasper, 1987) but these were too complex to analyse at the population level. Isoenzymes provided the first tool to demonstrate variation subdividing 35 isolates into four main zymodeme groups (Dardé *et al*., 1992). This analysis was most interesting as all the acute virulent isolates fell into zymodeme 1 showing a clear relationship between genotype and phenotype. Simultaneously, Sibley and Boothroyd (1992) analysed 28 isolates based on single nucleotide polymorphisms within known protein-coding genes and found that virulent isolates formed a single group. As more markers were developed it became clear that there was a restricted amount of variation between isolates (Dardé *et al*., 2007). Rapidly evolving neutral markers based on microsatellites (Ajzenberg *et al*., 2002; Blackston *et al*., 2001) or repeated elements (Hogdall *et al*., 2000; Terry *et al*., 2001) were developed to enable better resolution of the relationships between strains.

6.3.2. Dominant clonal lineages

There was considerable consensus among genotyping studies that the pattern of strain diversity revealed strong groupings with a restricted number of multi-locus genotypes retrieved from a wide geographical area (Dardé *et al*., 1992, Dardé, 1996; Sibley and Boothroyd, 1992). In an analysis of 106 isolates collected mainly from across Europe and North America, Howe and Sibley (1995) proposed that the parasite had a clonal population structure with three dominant lineages represented by strain types I, II and III. These relationships were confirmed in further multi-locus typing studies based on microsatellite markers (Ajzenberg *et al*., 2002), or on sequence polymorphisms within independent loci (Grigg *et al*., 2001; Lehmann *et al*., 2004). In the context of these studies, the

remarkably high level of conservation among strains was noted. A study of 18 unlinked polymorphic genes among reference type I, II and III strains three type found only two alleles at any one locus. Based on this biallelic variation across such substantial proportions of the genome it was proposed that the clonal lineages arose from a single cross between two ancestral parents, the so-called Adam (A) and Eve (E) strains (Grigg *et al.*, 2001). This hypothesis was strengthened by a study of the distribution of 250 single nucleotide polymorphisms (SNPs) across the 14 chromosomes of the parasite (Kahn *et al.*, 2005) but received definitive proof from genome-wide mapping of SNPs in the three type strains (Boyle *et al.*, 2006). This latter study produces a detailed map of the regions derived from the original parental strains within type I, II and III isolates.

The very detailed understanding of the clonal lineages allows us to look at genotype–phenotype relationships in great depth in these strains. However, so-called 'atypical' strains containing novel alleles are reported at low frequency in many studies (Ajzenberg *et al.*, 2004; Su *et al.*, 2003). Our understanding of the allelic diversity of these strains is much less detailed; nevertheless, novel genotypes are increasingly detected as the global collection of Toxoplasma isolates grows.

6.3.3. Biogeography and evolutionary history

Initial collections of isolates were biased towards Europe and North America and were predominantly associated with clinical disease in humans and domesticated species (Dardé *et al.*, 2007). Clonal genotypes predominate in this collection and initially were thought to be more widespread. This was due to studies based on typing at the SAG-2 locus, which can differentiate between the three clonal genotypes but is not useful in assessing wider allelic variation (Su *et al.*, 2006). Multilocus typing of isolates collected over a wider geographical and species range reveals greater diversity. Some of the first indications of this came from discovery of a group of strains derived from French Guyana that were associated with severe disease in humans. These isolates did not fit into the clonal pattern and had unusual isoenzyme and microsatellite profiles (Ajzenberg *et al.*, 2004; Dardé *et al.*, 1998). Since then, Toxoplasma strains with unusual genotypes have been recovered from Brazilian patients suffering from ocular toxoplasmosis and with AIDS (Ferreira *et al.*, 2008; Khan *et al.*, 2006). Further evidence that South American strains differ from the clonal types comes from genotyping of isolates in chickens from Brazil and Chile (Dubey *et al.*, 2006, 2007b) and from other species, including cats and dogs in Brazil (Pena *et al.*, 2008). As the number of South American isolates increases relationships are becoming clearer and clusters of 'South American' isolates are emerging. Lehmann *et al.*, 2004 compared variation at seven loci from approximately 50

Brazilian and North American isolates from domesticated species. Linkage disequilibrium was found at both locations but was lower in the Brazil indicating a higher degree of recombination between strains. Knowledge of the strain diversity elsewhere in the world is beginning to emerge. Studies in Africa have cited the presence of Clonal strain types (Lindstrom *et al*., 2008; Velmurugen *et al*., 2008) although other non-clonal genotypes have been reported (Lehmann *et al*., 2006). In Sri Lanka, type II and novel genotypes are reported (Dubey *et al*., 2007c) while in China novel genotypes have also been reported (Dubey *et al*., 2007a).

Multi-locus typing studies demonstrate strong biogeographical influences on the distribution and diversity of strains. Attempts to reconstruct the evolutionary history of these strains have been made. Su *et al*. (2003) generated comparative SNP data over introns of type I, II and II isolates. Based on the intron rate calculated for *Plasmodium* species, the divergence of the three clonal lineages was estimated at around 10,000 years ago. It is proposed that adaptation to oral transmission provided the basis for the unprecedented success of these strains. However, the timing also coincides with the expansion of humans and domesticated species so environmental change may also have provided a stimulus. Further analysis incorporating additional data from South American strains focused on the separation of North and South American strains (Khan *et al*., 2007). The analysis revealed some high-level resolution with strains falling into 11 haplotypes from four proposed ancestral lineages. The split is estimated as occurring 1 million years ago, interestingly the highly conserved chromosome 1a was associated with clonal strains in both North and South America giving rise to the suggestion that it is responsible for driving expansion of asexually transmitted lines (Khan *et al*., 2007; Sibley and Ajioka, 2008; Su *et al*., 2003).

6.4. GENETIC VARIATION AND STRAIN PARTITIONING

6.4.1. Distribution of strains

Improved understanding of the parasite population genetics has given insight into biogeographical variation in Toxoplasma but has it also contributed to evaluation of transmission? The earliest studies of genetic diversity reported a bias in the distribution of clonal strain types with type I strains being acute virulent, type II being more common in humans and type III isolated mainly from animal species (Howe and Sibley, 1995). If the overall abundance of strains is taken into account, the majority of isolates in circulation in Europe and North America fall into the type II lineage (Howe and Sibley, 1995; Howe *et al*., 1997). In contrast, type I strains are under-represented in strain collections and although they are

often detected by polymerase chain reaction (PCR) screening (Aspinall *et al.*, 2003). This question of bias in detection of parasite strains is difficult to resolve as it is likely that our ability to detect and isolate parasites may be determined by the burden. Genotyping can be used to evaluate the partitioning of strains, as, for example, found in a recent study comparing the genotypes of Brazilian isolates from cats with those previously reported in chickens and dogs. While a number of unique isolates were detected in single host species, many isolates fell into clonal lines that were geographically widespread and infected all three host species (Pena *et al.*, 2008). One important question for human health relates to whether strains are transmitted between humans and animals. Lindstrom *et al.* (2006, 2008) working in Uganda where re-activation of toxoplasmosis in AIDS patients is a serious problem, found the relative abundance of type I, II and III isolates in patients matched that of chickens from the same geographical area.

Progress is most likely to be made in studies that focus on a specific habitat such as the peridomestic environment. In a study of risk factors in the transmission of Toxoplasma to pigs, Lehmann *et al.* (2003) typed isolates from a group of 55 pigs and 165 mammals and birds. The infection prevalence was vey high in pigs (95%) with three genotypes represented. The majority of wild species were uninfected but the main genotype infecting pigs was also found in chickens and mice, and a second genotype was shared by pigs and chickens. The data suggest that oocysts excreted around pig sties were the most likely source of infection. In studies of congenital infection in sheep genotyping of parasite DNA via mobile genetic elements (MGE) PCR indicated the presence of a single parasite strain transmitted through lamb cord tissue (Duncanson *et al.*, 2001; Terry *et al.*, 2001). Recently, Dubey *et al.* (2007d) analysed the strain composition in isolates obtained from slaughter lambs and found considerable variation with 11 novel genotypes in addition to type II and III strains. These studies show the complexity of strain distribution at a local level. Parasite genotyping provides a tool to track infection but it is the depth of sampling that is critical to identification of transmission networks.

Perhaps the most comprehensively studied system is the transmission of Toxoplasma in marine mammals. Sea otters have a high prevalence of infection with *T. gondii* and can suffer from severe disease (Conrad *et al.*, 2005). Modelling the distribution of disease revealed that areas of high freshwater run-off were a significant risk factor for infection and animals as high-risk sites had a five to six times more likely to catch disease (Miller *et al.*, 2002). Genotyping of isolates showed an unusual polymorphism at a number of loci; this X allele was common in isolates from otters, however, random amplification of polymorphic DNA (RAPD) analysis indicates that there is further genetic variation within type-X strains (Conrad *et al.*, 2005). A recent survey of species in the watershed area demonstrates that

wild felids and canids have very high seroprevalence of toxoplasmosis in the area carry all four strain types (type I, II, I II and X). Furthermore, PCR screening led to profiling of strains in wild invertebrates and the detection of Toxoplasma via PCR in wild mussels (Miller *et al.*, 2008b). It is proposed that bivalves, which are eaten by otters, might accumulate the oocysts from the water and act as a transport host. This careful work outlines a potential cycle for Toxoplasma forging a link between terrestrial and marine species in maintaining the parasite. The type-X strain has also been reported from harbour seals and sea lions so it may be adapted to the marine environment (Conrad *et al.*, 2005). The caveat is that type-X may simply be a local geographical variant, and, if local clonal expansion is a feature of the parasite's epidemiology, considerable resolution is needed to assess diversity.

6.4.2. Recombination

An overview of Toxoplasma population structure indicates that there are differences in the importance of sexual cycle and that the potential for recombination varies (Khan *et al.*, 2007). Clear contrasts can be made between the strain composition between North and South America but it is possible that similar patterns exist on more local scales and are important determinants of transmission. One of the key questions relates to why the clonal lineages are so dominant and whether recombination occurs. There does not appear to be any specific block as experimental crosses have shown a normal frequency of recombinants (Su *et al.*, 2002). It has been argued that recombination is unlikely to occur in the field because the sexual stage is transient and a cat is not likely to ingest two strains simultaneously, but this also presumes that there will be no mixed infections in intermediate hosts due to strong immunity (Dardé *et al.*, 2007). In fact it has been demonstrated superinfection definitely can occur (Dao *et al.*, 2001) both in experimental studies and mixed infections have been detected in many hosts through isolation and genotyping studies (Ajzenburg *et al.*, 2002; Dubey *et al.*, 2007c; Lindstrom *et al.*, 2007). Early studies suggested that recombinant isolates were rare in nature, but this may in part be due to the fact that many studies used single locus typing. Recombinant isolates have been detected in a number of studies with multi-locus typing from Brazil (Cavelcante *et al.*, 2007; de Melo Ferreira *et al.*, 2006) and a natural type II/III recombinant strain from Uganda has recently been isolated (Lindstrom *et al.*, 2008). It is possible that some recombinant isolates currently go undetected due to the low coverage of markers and they may be more common that we at first perceived. It is important to detect recombination as hybrid strains may have different phenotype (Grigg *et al.*, 2001).

6.5. TOWARDS DEFINING TRANSMISSION NETWORKS

6.5.1. Epidemiological investigation

Considerable advances have been made in understanding the interactions between *Toxoplasma gondii* and its hosts. We have established that there is biological and genetic diversity between isolates and biogeographical differences in the parasite population structure. We now need to think in terms of ecosystem rather than in terms of the individual host species. To evaluate strain partitioning we need to consider sympatric studies where isolates derived from multiple hosts in the same environment are genotyped to identify and track the circulation of strains. Markers need to be appropriate to local background and this may require an initiative to sequence and map polymorphisms at high density from emerging strain types with novel alleles.

This ecosystem-based approach would aim to discriminate oocyst-induced infection from asexual transmission by analysing heterogeneity in the distribution of genotypes and testing the frequency of recombination. It would evaluate the importance of oral transmission by testing whether parasite strains map to tropic networks between hosts, and it would assess more widely the impact of vertical transmission in this parasite.

6.5.2. Genetic basis of disease

The high level of conservation seen among Toxoplasma strains is a great asset in investigating the genetic basis of disease. Close typing of isolates is possible due to the high density of SNPs from the Toxoplasma genome project (Su *et al.*, 2002). Genetic crosses between the main clonal strain types have already been used to great effect to identify genes such as ROP 18, which encodes a serine-threonine kinase associated with virulence (Taylor *et al.*, 2006). Natural recombinant strains provide a resource to examine phenotype–genotype associations and gain insight into disease mechanisms.

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CHAPTER 7

Parasites and Biological Invasions

Alison M. Dunn

Contents

Abstract

There is considerable current interest in the role that parasites can play in biological invasions. This review looks at the fate of parasites during a biological invasion and at their impact on native and invasive hosts, and asks whether parasites can mediate invasion success. An introduced species may lose its parasites as a result of the introduction and such release from its natural enemies may be an important factor determining invasion success. In addition, an introduced species may acquire parasites from its new environment or it may introduce novel parasites to hosts in the new range. As a result of local adaptation, parasites tend to have a differential

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effect on native versus invading hosts. The relative impact on the fitness of natives and invaders can be important for the outcome of an invasion and may, for example, reverse the pattern of competitive dominance seen in uninfected hosts. Parasites may mediate invasion success through their effect on host fitness and thus on host population growth and stability. Furthermore, by modifying host–host interactions (including competition and predation), parasites can be important factors that determine the success of an invasion and its impact on the recipient community.

7.1. INTRODUCTION

Biological invasions are global phenomena that can have dramatic impacts on biodiversity and community structure as well as economic implications for both natural and managed habitats (e.g., Lodge *et al.*, 2000; Pimentel *et al.*, 2001). There is growing interest in the role that parasites can play in biological invasions; through their effects on host fitness and by modifying interactions between native and invasive species, parasites can play a key role in determining the success of an invasion (e.g., Hatcher *et al.*, 2006; Prenter *et al.*, 2004). Furthermore, biological invasions provide an opportunity to investigate the effect of parasites on interactions between species.

This review looks at the role that parasites can play in biological invasions by asking what happens to the parasite fauna in the event of an invasion; how does this affect the success of the invader and how do parasites mediate the impact of an invasion on organisms in the invaded community? The first part of the review looks at the different possible outcomes of an invasion in terms of changes to the parasite fauna of the invasive species and the recipient community (Fig. 7.1). First, an introduced species (see Box 7.1) may lose its parasites as a result of the introduction, with this release from its enemies facilitating its growth and spread. Second, an introduced species may acquire parasites from its new environment. Finally, an introduced species may introduce novel parasites to its new environment. The second part of the review looks at the impact of parasitism on the success of biological invasions and asks how parasites can mediate the impact of an invader on the recipient community. Parasites can act directly on host fitness and thus on host population growth and stability. In addition, by modifying host–host interactions (such as predation or competition), parasites can be important factors that determine the success of an invasion and its impact on the recipient community. This review looks at examples from the literature, including work on crustacean invasions from the author's laboratory. Human parasitic infections are not covered in this article.

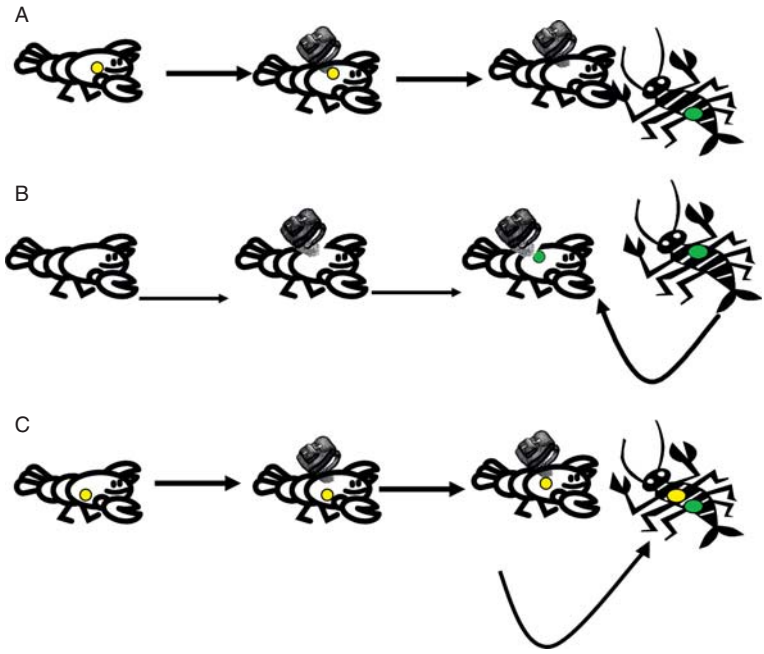

FIGURE 7.1 Possible outcomes for parasitism following the invasion of a new habitat. (1A) Enemy release; an invader may benefit from a reduction in parasite diversity and/or prevalence as a result of invasion. (1B) Parasite acquisition; an invader may acquire parasites in the new habitat. (1C) Parasite introduction; parasites introduced with the invader may infect novel host species in the new habitat.

7.2. ENEMY RELEASE AND PARASITE ACQUISITION

The process of introduction to a new habitat can lead to a change in parasite diversity and prevalence. An introduced species may lose parasites from its original range, and/or it may acquire parasites in the new habitat. Changes in parasite diversity, prevalence and burden may affect the establishment and spread of a parasite, and enemy release (see Box 7.1) may be a key factor in invasion success (Keane and Crawley, 2002; Torchin *et al.*, 2003). Animal introductions occur frequently and repeatedly. For example, it has been estimated that on any single day, ships transport over 3,000 species in their ballast water (Carlton and Geller, 1993) which may then be released into new habitats. In many cases, introduced species will fail to establish a viable population. However, in other cases, they may establish (stage 3 invaders; see Box 7.1; Colautti and MacIsaac, 2004) and become invasive (stages 4 and 5). Invading species may reach higher population densities than those

BOX 7.1 Glossary

Apparent competition Two species do not compete directly for resources but can cause a reduction in each other's population sizes via a shared enemy (predator, parasite) (Holt, 1977).

Dilution effect A situation where an alternative, resistant host creates a dilution effect, lowering the infection prevalence in the main host (Norman *et al*., 1999; Ostfeld and Keesing, 2000).

Enemy release The enemy release hypothesis proposes that the success of some invaders is a result of the scarcity of natural enemies (predators, parasites, etc.) in the new habitat (Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin *et al*., 2003).

Established species/stage 3 invaders A species with a self-sustaining population outside of its native range (Colautti and MacIsaac, 2004; Kolar and Lodge, 2001).

Indigenous species/native species A species that is found within its native range (Kolar and Lodge, 2001).

Intra-guild predation Predation among species that are also potential competitors (Holt and Polis, 1997; Polis *et al*., 1989).

Introduced species A species that has been introduced into a new habitat where it may or may not become established and spread (become invasive).

Invasive species/stage 4 and 5 invaders An introduced species that becomes established and spreads in the new habitat (Colautti and MacIsaac, 2004).

Local adaptation Parasites have higher fitness in local hosts with which they have a co-evolutionary history than in allopatric hosts (Dybdahl and Lively, 1995; Gandon, 2002; Kaltz and Shkoff, 1998).

Parasite-modified competition Two species compete with one another and a parasite (infecting one or both species) influences this interaction (Hatcher *et al*., 2006).

Propagule/founder population The group individuals of a species introduced into a non-native habitat. In some cases, there may be a single introduction. More commonly, introductions occur repeatedly over time.

Reservoir host An alternative host for the parasite that permits an increase in the abundance of a native parasite, thereby potentially reducing the population growth rate of the main, susceptible hosts (de Castro and Bolker, 2005).

found in their native habitat as well as reaching higher densities in comparison with similar species in the invaded habitat (Torchin *et al*., 2003). In addition, individuals of an invasive population are often larger

than their conspecifics in the native range (Grosholz and Ruiz, 2003; Torchin *et al.*, 2002). The enemy release hypothesis proposes that the success of an invader is related to a reduction in natural enemies (herbivores, predators and parasites) in the new range in comparison with the native range (Keane and Crawley, 2002; Torchin *et al.*, 2002; 2003).

During the introduction of a new species, there are a number of stochastic and selective pressures that are likely to lead to the loss of parasites (Torchin *et al.*, 2002). First, parasites may be lost as a consequence of subsampling. Any introduced population will be a sub-sample of the original population. As a result, invasive populations frequently experience a genetic bottleneck (e.g., Cristescu *et al.*, 2004; Muller *et al.*, 2002). This subsampling is also likely to lead to loss of parasites if infected hosts are not included and the likelihood of parasite loss will be greater for parasites at low prevalence in the source population (Colautti *et al.*, 2004; Drake, 2003). Second, during the process of translocation and colonisation, selective pressures may lead to the differential loss of parasitised (and so less fit) hosts, while favouring resistant host genotypes (Colautti *et al.*, 2004). Third, parasites may be lost from invading populations as a result of reduced transmission opportunities (Colautti *et al.*, 2004). Transmission depends on host density for many parasites (Anderson and May, 1986). If host densities are low during an invasion, this may lead to loss of parasites from the population. In addition, parasites that have complex life cycles may be lost if they arrive with their host, but then are unable to be transmitted to the next host in their life cycle. Finally, for some marine invaders, larval stages introduced in ballast water may not be infected by parasites that infect the adult-stage host (Lafferty and Kuris, 1996).

Enemy release may benefit the invader in two ways (Colautti *et al.*, 2004). The host may benefit through release from the regulatory pressure of the parasite (e.g., parasite-induced reduction in survival or fecundity). In addition, the loss of parasites may benefit the host if resources for defence are re-allocated elsewhere either through plasticity of the host or through evolutionary changes (termed the evolution of increased competitive ability; Blossey and Notzold, 1995). Although there is evidence for enemy release in a number of systems, few studies have tested the impact of parasite loss on host fitness and invasion success (Colautti *et al.*, 2004).

Drake (2003) explored theoretically the effect of enemy release and population size on the likelihood of invasion success. On the one hand, the likelihood of an introduced species becoming established should increase with propagule size (see Box 7.1). However, the larger the propagule size, the more likely it will be that parasites will also be introduced with and become established in invading population. Hence, Drake predicted that enemy release is unlikely to affect the probability of establishment. However, field surveys and meta-analyses of parasitism in native and invasive populations provide many examples of enemy release in

plants (e.g., Liu and Stiling, 2006; Mitchell and Power, 2003) and animals (e.g., Torchin *et al*., 2003). Tests for enemy release fall into two categories (Colautti *et al*., 2004); community studies that compare parasitism in invading species and their indigenous (see Box 7.1) counterparts and biogeographical studies that compare parasitism of an invasive species in its native and invasive habitat. Both approaches explain the importance of enemy release for the success of an invader, while the community approach may also develop our understanding of the interaction of invader with indigenous species in the invaded community.

7.2.1. Community studies

A number of studies compare patterns of parasitism in native versus invading hosts in the invaded habitat. Parasites from the invaded habitat may be restricted to native hosts, or may have also been acquired by the invading species. In addition, novel parasites introduced by the invader may remain host specific, or may be introduced to native species. In a number of studies, a pattern of higher parasite diversity is seen in the native species in comparison with the invasive species. This pattern is likely to result from parasite specificity and local adaptation (see Box 7.1) to the host. However, patterns of parasite prevalence vary with some studies reporting higher prevalence in the native species, while fewer report higher incidence in invaders.

In the Mediterranean, the invasive American brine shrimp *Artemia franciscana* is replacing the native species *A. parthenogenetica* and *A. salina* (Amat *et al*., 2005). A comparison of parasite diversity and distribution in the native and invasive species of the Mediterranean indicated that the invader was susceptible to only six of the 10 species of cestode that utilise native brine shrimps as intermediate hosts in the Mediterranean. Furthermore, pair-wise comparisons revealed that parasite prevalence was higher in the native host in 28 out of 30 populations (Georgiev *et al*., 2007). *A. franciscana* can cause the rapid extinction of the native *A. salina* owing to its higher reproductive rate and competitive ability (Browne and Halanych, 1989). It is probable that differential infection by cestodes increases the rate of replacement (Georgiev *et al*., 2007).

Studies of mollusc invasions also provide evidence that indigenous hosts suffer higher parasite diversity. For example, an invasive lineage of the clonal snail *Melanoides tuberulata* occurs in sympatry with an indigenous lineage in Lake Malawi (Africa). Field surveys show that the invader has lost trematode parasites from its native range (East Asia), and that it is resistant to parasites infecting the native lineage in the new habitat (Genner *et al*., 2008). The invasive morph was numerically dominant to the indigenous morph and its distribution is expanding; a situation that may be facilitated by its lack of parasites.

Similarly, the invasive mud snail *Batillaria cumingi* appears to be resistant to several native trematode species that infect the native *Cerithidea californica* in North America (Torchin *et al.*, 2005). Overall prevalence of trematodes did not differ in invasive versus native snails. However, while the native snail *Cerithidea californica* was host to 10 species of trematode, the Japanese invader *Batillaria cumingi* was infected by only one parasite species that was thought to have been introduced along with its host and which is able to utilise native fish and birds as definitive hosts.

Our laboratory has examined parasitism in amphipod invasions in the United Kingdom. In Northern Ireland, the native amphipod *Gammarus duebeni celticus* is under threat from the invasion by the European *G. pulex*, and the American *G. tigrinus*. The native *G. duebeni celticus* suffers from a higher parasite diversity than do the invading species. Five parasite species were detected of which three were shared, but two, the microsporidian *Pleistophora mulleri* and the acanthocephalan *Polymorphus minutus*, were specific to the native host (Dunn and Dick, 1998; MacNeil *et al.*, 2003a; 2003b; 2003c). Parasite prevalence and burden were also higher in the native species for two of the shared parasites (Fig. 7.2).

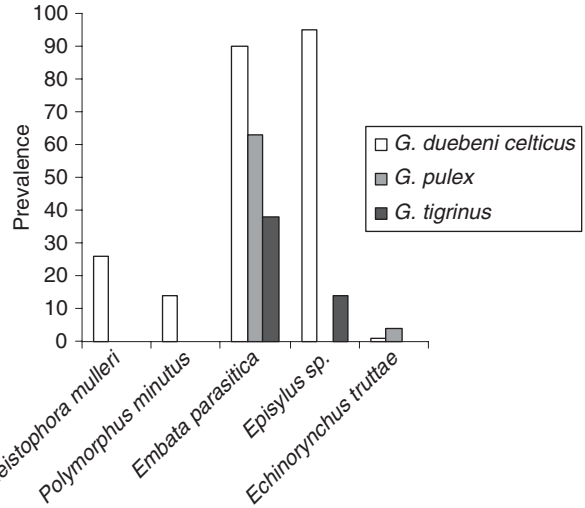

FIGURE 7.2 A summary of parasite prevalence in indigenous (*Gammarus duebeni celticus*) versus invading (*Gammarus pulex, G. tigrinus*) in Northern Ireland. *Notes: The microsporidian* Pleistophora mulleri *and the acanthocephalan* Polymorphus minutus *were specific to the native host. Prevalence of the rofiter* Embata parasitica *and the protozoan* Epistlyus *sp. occur at higher frequencies in the native species, while the acanthocephalan* Echinorynchus truttae *had a higher % of individuals infected in the invading* G. pulex. *Data summarised from MacNeil* et al. *(2003a; 2003b; 2003c); Dunn and Dick (1998).*

7.2.2. Biogeographical studies

Biogeographical studies of enemy release compare patterns of parasitism in hosts from their native habitat versus their invasive habitat (Colautti *et al.*, 2004). There are a number of detailed studies of parasitism for single species of invaders as well as meta-analyses that use the literature to test for patterns of parasitism in invaders.

A review of enemy release in marine invaders, including molluscs, crustaceans, ctenophores, and echinoderms (Torchin *et al.*, 2002), found that the species richness of parasites in the native range (mean number of species = 6) was three times higher than that in the invasive range. Although more studies were available of parasitism in the native range, the authors concluded that sampling effort was unlikely to explain this pattern as large numbers of hosts were examined in each region. Although parasite diversity was lower, the average parasite prevalence in the introduced range was higher than in the native range.

Similarly, in a key meta-analysis of introduced species, Torchin *et al.* (2003) compared parasite diversity and prevalence in the native and invasive ranges of 26 species, including molluscs, crustaceans, fish, birds, amphibians, reptiles and mammals. They found that while an average of 16 parasite species were found in native populations, on average only seven species of parasite were present in the invasive range; of these, three had been introduced with the invader, while four had been acquired from the new habitat. However, for those parasites that were introduced with the host, there was no difference in prevalence in the native and invasive range.

Other studies have looked at single examples of invaders. For example, the invasive mosquito *Aedes albopictus* in North America has a reduced prevalence of the gregarine *Ascogregarina taiwanensis* in the years following introduction, which may increase its competitive impact on the native *Ochleoratatus triseriatus* (Aliabadi and Juliano, 2002; Juliano and Lounibos, 2005). Similarly, native populations of the rabbit fish *Siganus rivulatus* in the Red Sea were found to have a higher diversity of parasites (24 species) than did invasive Mediterranean populations (nine species, Diamant 1989, 1999; reviewed in Pasternak *et al.*, 2007). Although parasite diversity was lower in the invasive rabbit fish populations, studies of a single species of flatworm, *Polylabris cf. mamaevi* revealed that prevalence and abundance were three times higher (Pasternak *et al.*, 2007). A similar pattern of infection was found in the invasive tree frog *Eleutherodactylus coqui* (Marr *et al.*, 2008). Frogs in the invasive Hawaiian range had a lower diversity of parasites (two species) than that found in the native range (eight species). However, there was no difference in overall parasite prevalence and one species of cestode (*Cosmocerca* sp.) that was indigenous to the invaded habitat occurred in much higher prevalences in the invader than in the native frogs in the new habitat.

A pattern of decreased parasite diversity has been reported for many invaders in their new range. However, for those parasites that were introduced with the host, or acquired in the new range, prevalence may be similar to or higher than that in the native hosts. This indicates that, if parasitised hosts are included in the founder population, these parasites are able to sustain transmission and even increase in prevalence. This pattern would fit with a pattern of enemy release as a result of sub-sampling, rather than a result of selective loss of infected hosts (Torchin *et al*., 2002). Furthermore, many invaders achieve higher population densities in the new range (Marr *et al*., 2008; Torchin *et al*., 2003) which will facilitate transmission to new hosts (Anderson and May, 1986).

Many of the studies that provide support for the enemy release hypothesis examine parasite diversity and prevalence in the introduced range of the host in comparison with that in the native range of the host (e.g., Torchin *et al*., 2003). However, there are two potential sources of bias here (Colautti *et al*., 2004). The data may be affected by differences in the research effort, with native populations likely to have been more extensively studied, leading to an underestimate of parasites in the invasive range, particularly if an invasion is recent. Sampling effort should therefore be taken into account, as for example in Torchin's (2003) meta-analysis of enemy release. In addition, many studies of enemy release compare parasitism in the native host range versus the invasive range. However, this is not necessarily biologically realistic. Invading propagules represent a subset of animals from the native range and are unlikely to be a random sample from across the range, but are more likely to come from a sub-set or even a single population within the native range. Colautti *et al*. (2004) termed loss of parasites as a result of sub-sampling from the native range 'apparent release' and predicted that this loss of parasites would have no impact on the introduced population. They termed the loss of parasites under the selective pressures of translocation and establishment 'realised release' and proposed that this would be more likely to increase host fitness. Colautti *et al*. (2004) propose that tests of enemy release should compare parasitism in the invasive range with that in the likely source population. This requires that the probable source of infection is identified from either historical data or host population genetics and that average parasite diversity across several populations be compared.

A few studies have taken into account the risk of sampling bias and compared parasites in invasive and source habitats. In a key study of starlings in their native Eurasian and invasive North American habitat, Colautti *et al*. (2005) measured both apparent and realised reduction in helminth parasites. A comparison of parasite diversity in the native and invasive range revealed apparent enemy release. However, there was considerable geographic heterogeneity in parasite diversity in the native range. Similarity between parasites in North America suggested that the

founder starlings originated in the United Kingdom, and this is in accord with historical records. The authors, therefore, compared parasitism in the source and invasive populations and found no difference in the mean parasite diversity in the United Kingdom versus North America. The starlings appeared to have lost parasites from the native range (13/20 parasite species found in the United Kingdom were present in North America), and to have acquired parasites from the new habitat; 17 parasite species infecting North American starlings were not recorded in Eurasia. This study highlights the importance of looking at the source of the invasion when testing for enemy release.

Two recent studies have tested for enemy release in populations of amphipod invaders, taking into account the source of the invasion (Wattier *et al.*, 2007; Slothouber-Galbreath *et al.*, In press). Both studies used molecular sequence data to investigate the genetic diversity of both the hosts and their microsporidian parasites. Crustacea are important invaders globally (Cristescu *et al.*, 2004; Dick and Platvoet, 2000; Lodge *et al.*, 2001) and can lead to the extinction of native species (Dick and Platvoet, 2000) as well as having declines in fish productivity (Kelly and Dick, 2005). Microsporidia are important parasites of crustaceans. They are widespread and diverse in amphipods (Terry *et al.*, 2004) and so provide a good model to look at enemy release. Furthermore, microsporidia can utilise both horizontal and vertical transmission routes (transovarial transmission from mother to offspring; Dunn *et al.*, 2001). While the transmission of horizontally transmitted parasites is density dependent, vertical transmission does not depend on host density. Furthermore, vertically transmitted microsporidia are typified by low virulence (Bandi *et al.*, 2001; Dunn *et al.*, 2001) as they depend on successful host reproduction for their transmission to the next host generation. Thus, although vertically transmitted parasites may be lost from invasive populations due to sampling effects, they are unlikely to be lost as a consequence of reduced fitness of infected hosts in the founder populations or as a consequence of low host density. This has led us to predict that vertical transmission is an effective mechanism of dispersal over long distances or in patchy habitats (Dunn *et al.*, 2001) and that vertically transmitted parasites should be less likely to be lost during the process of invasion (Slothouber-Galbreath *et al.*, 2004). Furthermore, a number of vertically transmitted microsporidia cause sex ratio distortion of the host (Dunn *et al.*, 2001; Terry *et al.*, 2004). Overproduction of females under parasitic sex ratio distortion will cause a higher rate of population increase (Hatcher *et al.*, 1999). Hence, we predict that infection by a parasitic sex ratio distorter may increase the likelihood of an initial invasion (Slothouber-Galbreath *et al.*, 2004). Such sex ratio distortion may not persist in the longer term, however, as arthropods have been shown to evolve strategies to avoid sex ratio distortion, for example, through

suppression (Hornett *et al.*, 2006), mate choice (Kelly *et al.*, 2001) or sperm competition (Dunn *et al.*, 2006).

The Ponto-Caspian invader *Dikerogammarus villosus* is invading West Europe via inland canals and waterways and threatens aquatic invertebrate diversity (van Reil *et al.*, 2006). A comparison of microsatellite diversity in the invasive and probable source populations (based on waterway geography) revealed no evidence for a genetic bottleneck in the invasive population, suggesting a massive invasion and/or recurrent invasions via inland waterways (Wattier *et al.*, 2007). There was no evidence for enemy release. A single species occurred in both the native and invasive range, while a further four species were found in the invasive range and may have been acquired from native species in the new habitat (Wattier *et al.*, 2007).

In contrast with *D. villosus*, a recent study (Slothouber-Galbreath *et al.*, In press) has shown that the invasive North American amphipod *Crangonyx pseudogracilis* has suffered a post-invasion genetic bottleneck since its introduction to Europe. The reduction in host diversity in the invasive range suggested a single area of origin for the invasion. Phylogenetic analysis revealed diverse parasites in the native range with parasite distribution structured within that of the host. However, despite the host bottleneck, we found no evidence for enemy release when we compared the average number of microsporidian species in the invasive populations versus the probable source population. Furthermore, the two parasite species that were introduced to the invasive range are vertically transmitted by their host. In the invasive range, *Fibrillanosema crangonycis* was found in all populations and at high prevalences. This parasite causes feminisation of the host (Slothouber-Galbreath *et al.*, 2004), leading to the conclusion that the introduction of this parasite with the host may facilitate population growth and invasion through its effect on host sex ratio.

In contrast with these observations of *C. pseudogracilis* parasites, the single species of parasite found in both native and invasive populations of the invasive amphipod *Dickerogammarus villosus* was horizontally transmitted (Wattier *et al.*, 2007). However, it is interesting to note that two of the parasites acquired by this invader in its new range are related to *Nosema granulosis*, another vertically transmitted microsporidian that causes feminisation in several amphipod hosts (Terry *et al.*, 2004). It is, therefore, interesting to speculate that acquisition of this parasite could facilitate the on-going invasion of *D. villosus.*

7.3. INTRODUCED PARASITES

When a parasite is introduced with its invasive hosts (see Box 7.1), then there are opportunities for transmission of the infection to novel hosts in the new habitat. For example, the invasive American grey squirrel *Sciuris*

carolinensis has replaced the native red squirrel *S. vulgaris* throughout most of England and Wales over the last 70 years (reviewed in Tompkins *et al.*, 2003). The replacement is mediated in part through competition for resources (Gurnell *et al.*, 2004). However, the invading grey squirrel has also introduced a parapox virus, which causes high mortality of the native red squirrel *S. vulgaris* (Rushton *et al.*, 2000; Tompkins *et al.*, 2003). Models predict that this virus has played a role in red squirrel exclusion and that the rate of decline of the red squirrel is much greater (around 20 fold) when the virus is present in the population (Rushton *et al.*, 2000, 2006; Tompkins *et al.*, 2003). The fungal parasite *Batrachochytrium dendrobatidis* has been implicated in the decline and extinction of several species of amphibian worldwide. Detection of the fungus in introduced American bullfrogs (*Rana catesbeiana*) worldwide suggests that these declines in native amphibians may result from introduction of the fungus to native species by farmed and feral bullfrogs (Garner *et al.*, 2006).

In aquatic habitats, the introduction of novel parasites along with their hosts can affect both wild populations and aquaculture. The United Kingdom has a single species of native crayfish, *Austropotamobius pallipes*, which is considered a keystone species in freshwater habitats (Holdich, 2003). The native crayfish used to be widespread in the United Kingdom. However, the native species is being replaced by the American signal crayfish *Pacifastacus leniusculus* in many areas of England and Scotland. This replacement is mediated by crayfish plague (caused by the fungus *Aphanomyces astaci*). The parasite is thought to have been introduced in the 1970s with the invasive American crayfish (Holdich, 2003; Kemp *et al.*, 2003). The parasite is asymptomatic in the American crayfish, which transmits it to the native species and, since the 1980s, many native populations have been eliminated by the parasite. As a result, populations of *A. pallipes* are now restricted to central and northern England (Holdich, 2003). Similarly, the monogenean, *Gyrodactylus salaries*, was introduced with fish stocks and subsequently infected wild salmon stocks in Norway where it causes mortality (Johnsen and Jensen, 1991; Torchin *et al.*, 2002). The swim bladder nematode *Anguillicola crassus* has been introduced from Asia to America and Europe where it causes mortality in native eel populations (Barse and Secor, 1999; Taraschewski, 2006).

7.4. PARASITE–HOST ADAPTATION

As a result of biological invasions, parasites are likely to encounter new host species whether through introduction of a new host that is naïve to the parasite, or through introduction of a parasite to a new habitat along with an invader. Local adaptation theory predicts that selection should

favour a parasite that specialises on common host genotypes. Parasite–host co-evolution should, therefore, result in lagged cycles of parasite and host gene frequencies (Gandon and Michalakis, 2002; Nee, 1989). This tracking of common host strains by parasites should lead to local adaptation of the parasite; parasites should have higher fitness in local hosts with which they have a co-evolutionary history, than in hosts from other populations (Dybdahl and Lively, 1995; Gandon, 2002; Kaltz and Shykoff, 1998). Local adaptation was demonstrated in a classic reciprocal transfer experiment with the snail *Potamopyrgus antipodarum* and its trematode *Microphallus* sp., (Lively and Dybdahl, 2000), which demonstrated that parasites were more infective to snails from sympatric populations than to snails from allopatric populations.

In the context of an invasion, the parasite may encounter a new host from a different species, thus breaking the tight linkage of parasite and host genotypes considered in models of local adaptation. Nonetheless, invasions provide an interesting situation in which to compare parasite fitness in hosts with which they have a co-evolutionary history with fitness in novel host species, and local adaptation theory provides a framework in which to consider the impact of parasites on invasion outcomes. First, if an invader acquires parasites in its new habitat then, given the absence of a co-evolutionary history between the parasite and its new hosts, Moret *et al.* (2007) predicted that parasites should have lower fitness in the invading than the native host. In contrast to this argument, however, Colautti *et al.* (2004) predicted that invaders should be more susceptible to native enemies, either because they are naïve to the parasites, or because genetic bottlenecks experienced during invasion might reduce the ability to evolve disease resistance. Second, invading hosts may bring parasites with them, which are then transmitted to novel hosts in the invaded range. Here the parasite should be adapted to the invader and should show lower fitness in the native hosts.

There are a number of studies that look at the impact of parasites on native and invading hosts. However, the focus tends to be on the impact of the parasite on host fitness and it is not simple to relate this to parasite fitness. For instance, a virulent parasite would be considered well adapted to its host if it led to host mortality and consequent parasite transmission. However, parasite-induced host morality in the absence of transmission is disadvantageous for both host and parasite (Bull, 1994; Dunn *et al.*, 1995).

Studies of the effect of native parasites on invasive species tend to support the hypothesis that the parasite should be locally adapted to the native host (Moret *et al.*, 2007). For example, the native acanthoceplanan *Pomphorhynchus laevis* infects both the native amphipod *Gammarus pulex* and the invader *G. roeseli* in France. Rigaud and Moret (2003) found that phenoloxidase enzyme activity (which controls encapsulation of

the parasite) was lowered in the native *G. pulex* in response to the infection, but that infected *G. roeseli* (invader) had elevated activity, suggesting that the parasite evades the immune responses of their local host, but not of the invader. Similar evidence has been found in invading gastropods and beetles. In their study of the snail *Melanoides tuberulata* (see Section 7.2.1). Genner *et al*. (2008) found that an invasive lineage was resistant to trematodes that infect and cause castration and gigantism of the native lineage in Lake Malawi. Firlej *et al*. (2005) found that parasitoid *Dinocampus coccinellae* was maladapted to the invasive ladybird *Harmonia axyridis* but successfully parasitised the native *Coleomegilla maculata lengi* in Canada.

7.5. THE IMPACT OF PARASITISM ON BIOLOGICAL INVASIONS

In the case of invasions, parasites may be specific to one species of a native-invader pair, or may affect both species. The impact of a shared parasite can be complex. For example, an invader may benefit the native host if it creates a dilution effect (see Box 7.1) whereby transmission to the resistant, invasive host lowers the infection prevalence in the main, native host (Norman *et al*., 1999; Ostfeld and Keesing, 2000). Conversely, the introduction of an alternative (invading) host, rather than diluting the effects of the parasite, may act as a reservoir for infection, a factor that may be exacerbated by high densities of the invading hosts. The arrival of an alternative host could thus result in an increase in abundance of the native parasite, resulting in reduced population growth of susceptible hosts (Holt and Lawton, 1993). Such 'apparent competition' (see Box 7.1) between host species can result in the extinction of the host suffering the greatest negative impact of the shared parasite (Holt and Lawton, 1993).

7.5.1. Invaders and parasite dilution

Kopp and Jokela (2007) compared the impact of parasitism by *Microphallus* sp. on native and invading snails in mixed versus single species populations in New Zealand. In the native *Potamopyrgus antipodarum*, parasite prevalence was twice as high in single species populations as in populations where *P. antipodarum* was kept in sympatry with the invader. This dilution effect may contribute to the co-existence of native and invading snail in New Zealand (Kopp and Jokela, 2007). Similarly, in Ireland, prevalence of the bacteria *Bartonella birtlesii* and *Bartonella taylorii* declined in populations of the wood mouse *Apodemus sylvaticus* as densities of the invading bank vole *Clethrionomus glareolus* increased (Telfer *et al*., 2005). The parasite is transmitted by fleas and feeding by the fleas

upon the resistant bank vole led to a decline in infection in *A. sylvaticus*, hence the rate of invasion may be slowed (Telfer *et al.*, 2005).

7.5.2. Invaders as reservoirs for infection

The role of apparent competition in a biological invasion was first investigated by Settle and Wilson (1990) who undertook field experiments to look at competition between the native leafhopper, *Erythroneura elegantula*, and the invasive variegated leafhopper, *E. variabilis.* Direct competition between the species was not important for the invasion. However, the establishment of the invasive species provided a reservoir host (see Box 7.1) for the parasitoid *Anagrus epos* leading to an increased parasitoid population. As the native leafhopper suffered higher attack rates by the parasitoid *Anagrus epos*, the parasite shifted the competitive balance towards a strong advantage for the invader.

The grey squirrel acts as reservoir for the parapox virus responsible for the decline of the native red squirrel (see Section 7.3). While the virus leads to death of the native red squirrel (Tompkins *et al.*, 2002), the virus is asymptomatic in grey squirrel, which could therefore act as a reservoir (Sainsbury *et al.*, 2000).

The invasive North American signal crayfish, *P. leniusculus*, may act as a reservoir for parasites that are detrimental to the native white clawed crayfish, *Pacifastacus leniusculus*, in the United Kingdom. Crayfish plague is shared by the two species as discussed earlier. In addition, we have presented molecular evidence that the microsporidian *Thelohania contejeani* has been acquired by the invader (Dunn *et al.*, 2008). The impact of this shared parasite on the invasion by the signal crayfish and its extirpation of the native is currently under investigation. On the one hand, the spread of the invader might be slowed directly as a result of parasite acquisition, if the parasite causes mortality and/or reduced productivity of the invader. However, preliminary data suggest that the infection causes lower mortality in the invader than in the native. *T. contejeani* prevalence was high (26–75%) in the *P. leniunsculus* populations studied (Dunn *et al.*, 2008) and the signal crayfish occurs at much higher densities than its rival (Peay, personal communication). These factors suggest that *P. leniusculus* may act as a reservoir for *T. contejeani.*

7.5.3. Parasite modification of native-invader interactions

The majority of studies of biological invaders focus on their competitive or predatory interactions with native species. However, parasites can modify these interactions (Hatcher *et al.*, 2006, 2008) with important potential effects on invasion (Hatcher *et al.*, 2006). Parasites may mediate host–host interactions both through their effects on host fitness

(and hence host population density). They may also act on the host through modification of native–invader interaction strengths such as reducing a host's competitive ability (Aliabadi and Juliano, 2002), increasing exposure to predation (Mouritsen and Poulin, 2005) and increasing susceptibility to intra-guild predation (see Box 7.1; MacNeil *et al*., 2003a; 2003d; Hatcher *et al*., 2008).

Studies of competition between *Aedes ablopictus* and *Ochlerotatus triseriatus* demonstrate that competition is modified by the gregarine parasite *Ascogregarina taiwanensis;* infected *A. albopictus* have a lower effect on the survivor ship of *O. triseriatus* than do uninfected individuals (Aliabadi and Juliano, 2002). During the initial years following introduction of *A. albopictus* to North America, infection is low and this escape from parasites may make *A. albopictus* more competitive during the initial phase of invasion (Juliano and Lounibos, 2005).

Parasites, particularly those that are trophically transmitted to the definitive host, may increase the risk of predation. This can mediate invasion success if native and invading hosts are differentially affected. For example, the native acanthoceplanan *Pomphorhynchus laevis* infects both the native *Gammarus pulex* and the invader *G. roeseli* in rivers in Eastern France. The parasite alters the phototactic response of the native host, making it swim to the surface where it is vulnerable to predation by the definitive fish host (Cezilly *et al*., 2000; Tain *et al*., 2006). In contrast, the parasite does not manipulate the behaviour of the invasive host (Bauer *et al*., 2000; Tain *et al*., 2006). Similarly, the invasive American brine shrimp *Artemia franciscana* has acquired cestode parasites from natives in its new Mediterranean range. However, while these parasites cause colour change and reversed phototaxis in the native species (Sanchez *et al*., 2006), parasite manipulation of anti-predator behaviour has not been observed in the invader (Georgiev *et al*., 2007).

Intra-guild predation refers to predation among species that are also potential competitors (Holt and Polis, 1997; Polis *et al*., 1989) and often occurs among taxonomically related species (Dick *et al*., 1993). It is a key factor in native–invasive interactions and recent work has illustrated the impact of parasitism on intra-guild predation (Hatcher *et al*., 2008; MacNeil *et al*., 2003a, 2003d). We have shown theoretically that shared parasitism can increase the conditions under which intra-guild predators may co-exist (Hatcher *et al*., 2008). Co-existence is possible if the parasite exerts a greater deleterious effect on the 'stronger' species in terms of the combined effects of competition and predation. In some cases, we predict that shared parasitism can lead to a reversal of numerical dominance with the 'weaker' species attaining the higher population size. Thus, the parasite can be considered a keystone species determining the outcome of the association.

We have used amphipod invasions in the United Kingdom to investigate empirically the impact of parasites on host–host interactions,

in particular intra-guild predation. In Ireland, the native *Gammarus duebeni celticus* is being replaced by the invasive *Gammarus pulex*. Two other invaders also occur, *G. tigrinus* and *C. pseudogracilis*. Behavioural observations reveal a hierarchy of competition and intra-guild predation; the native *G. duebeni celticus* is larger than *G. tigrinus* and *C. pseudogracilis* and is a stronger competitor and predator upon these invaders. *G. pulex* is the largest amphipod in these communities and is a stronger competitor and predator than the native. However, this predation hierarchy is strongly influenced by parasitism.

The microsporidian *Pleistophora mulleri* infects only the native host, *G. duebeni celticus*. It causes no direct mortality or reduction in fecundity, but shows cryptic virulence when we look at interactions with invasive species (Fig. 7.3, MacNeil *et al*., 2003a). Field enclosure experiments revealed that *P. mulleri* had no impact on *G. duebeni celticus* survival, either in single or mixed species populations (Fig. 7.3A,B). However, the parasite reduced the ability of *G. duebeni celticus* to resist invasion by *G. tigrinus*; survival of *G tigrinus* was less than 10% over 2 weeks in sympatry with uninfected natives, whereas the invader co-existed with infected natives (Fig. 7.3B). Laboratory experiments confirmed that co-existence was facilitated by a strong reduction in the ability of infected *G. duebeni celticus* to predate the smaller invader. Similarly, infected *G. duebeni celticus* were less predatory on the smaller *Crangonyx pseudogracilis*, while they suffered higher levels of intra-guild predation by the larger invader.

The acanthocephalan *Echinorynchus truttae* also mediates invasion success. This acanthocephalan is more common in the invader *G. pulex* than in *G. duebeni celticus* and affects host behaviour. Infection reduced the predatory impact of the invasive *Gammarus pulex* on *Asellus aquaticus* (Fielding *et al*., 2003) and led to increased activity and photophilic behaviour, (MacNeil *et al*., 2003c), a trait that is likely to lead to greater predation by fish. Finally, *E. truttae* modified intra-guild predation between the native and invading amphipod. While the parasite had no direct effect on *G. pulex* survival, it caused a reduction in its predatory impact on the native species, so increasing its ability to co-exist with the larger invader (MacNeil *et al*., 2003b).

7.6. WIDER COMMUNITY EFFECTS

The replacement of a native species by an invader is known to have wide reaching effects on the community (Pimental *et al*., 2001). However, species replacements may also cause changes in parasite diversity and distribution, particularly for species that are host specific. A change in patterns of parasitism may in turn affect host population dynamics and of course

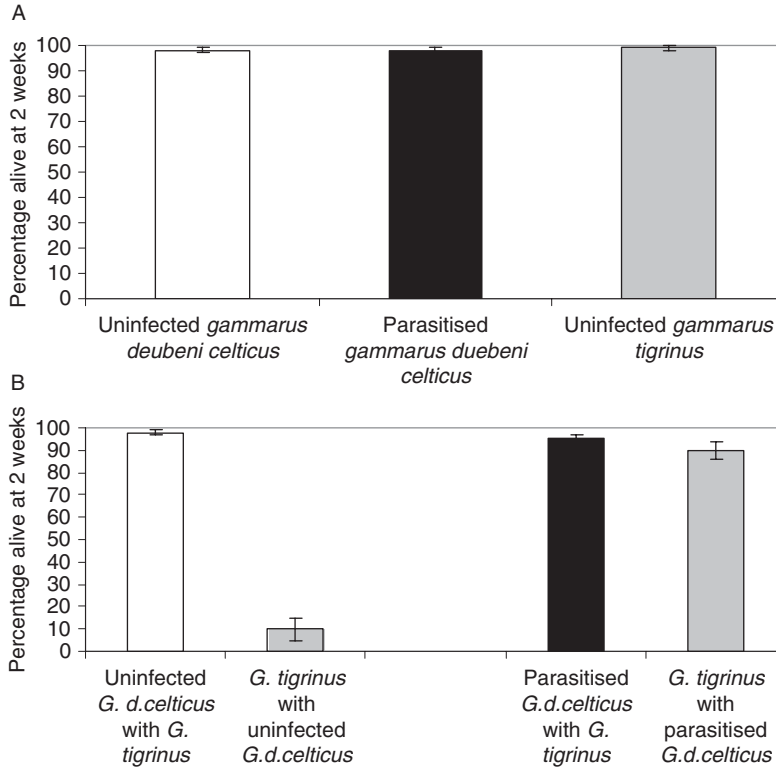

FIGURE 7.3 The effect of parasitism by *Pleistophora mulleri* on the survival of the native *Gammarus duebeni celticus* and the invasive *G. tigrinus* in field enclosures. *Mean percentage (+/− standard error of the mean (SE), data back transformed) survival at 2 weeks for animals in single (1A) and mixed (1B) species populations. Infection with* P. mulleri *did not affect the survival of* G. d. celticus *in either single or mixed species populations (Fig. 7.3A and B). However, infected animals were less able to predate the smaller invasive* G. tigrinus. *This led to higher survival of the invader when in sympatry with infected* G. d. celticus *than when in sympatry with uninfected* G. d. celticus *(Fig. 7.3B). Data from MacNeil et al. (2003a).*

the population dynamics of their prey and predators. The impact on the community may be particularly complex for parasites that have an indirect life cycle. For example, invasions of the mud snail *Batillaria cumingi* cause long-term decline and extinction of the native snail *Cerithidea californica* in North America. The native species is intermediate host to at least 10 species of trematode that have not been found in the invader (Torchin *et al.*, 2005). These trematodes have an indirect life cycle involving a shore bird definitive host, a snail first intermediate host and a second

intermediate host, which may be a mollusc, crustacean or fish. Torchin *et al*. (2005) speculate that extinction of the native snail host, and the consequent extinction of several species of trematode parasite, could affect several aspects of the community. Trematode extinctions might remove regulatory effects from the population dynamics of the various molluscs and crustaceans that are second intermediate hosts. In turn, shore bird populations may benefit indirectly if this increases their food availability, as well as benefiting directly from the absence of trematodes.

Similarly, populations of the invasive American brine shrimp *Aretmia franciscana*, which are replacing native species in the Mediterranean, suffer a lower diversity, prevalence and intensity of cestode infection than do native host populations. Furthermore, the behavioural manipulations and colour changes observed to enhance trophic parasite transmission in native shrimps are not induced in the invader (Georgiev *et al*., 2007). The lack of host manipulation will reduce the numbers of red brine shrimps at the water surface leading to a decline in parasite transmission and prevalence. The reduced parasite prevalence and host manipulation is also likely to reduce the foraging intake of water birds that feed on red brine shrimps (Georgiev *et al*., 2007).

7.7. FUTURE CHALLENGES

The direct effects of parasites on their host populations are well studied both theoretically and empirically. However, less is known about how patterns of parasitism change during an invasion and how these parasites affect population dynamics in the context of a biological invasion. Enemy release provides an explanation for the establishment and spread of biological invaders with numerous empirical examples. Future studies of enemy release should take into account possible biases inherent in large-scale geographical studies as well as including complementary studies of the impact of changes in parasitism on the outcome of an invasion (Colautti *et al*., 2004). Although parasites may be lost, invasions can also lead to novel situations of shared parasites and the presence of an alternative host has the potential to change the impact of a parasite on host population dynamics. As well as density-dependent effects, parasite modification of native–invader interactions is a burgeoning field (e.g., Hatcher *et al*., 2006) that is likely to elucidate the mechanisms underpinning biological invasions. It is important to consider the impact of parasitism on novel predatory and competitive interactions between native and invasive host species. Finally, there is a need to look beyond native–invader–parasite interactions to the wider effects of parasites on community diversity and structure.

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CHAPTER 8

Zoonoses in Wildlife: Integrating Ecology into Management

Fiona Mathews

Contents

Abstract

Zoonoses in wildlife not only play an important ecological role, but pose significant threats to the health of humans, domestic animals and some endangered species. More than two-thirds of emerging, or re-emerging, infectious diseases are thought to originate in wildlife. Despite this, co-ordinated surveillance schemes are rare, and most efforts at disease control operate at the level of crisis management. This review examines the pathways linking zoonoses in wildlife with infection in other hosts, using examples from a range of key zoonoses, including European bat lyssaviruses and

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bovine tuberculosis. Ecologically based control, including the management of conditions leading to spill-overs into target host populations, is likely to be more effective and sustainable than simple reductions in wildlife populations alone.

8.1. INTRODUCTION

Parasites and pathogens in wildlife are a natural part of biodiversity. An abundance of theoretical studies indicate that they have key roles in ecological and processes, including the regulation of population size (Anderson and May, 1979; May and Anderson, 1979) and the maintenance of genetic diversity (May and Anderson, 1983; Read *et al.*, 1995). Occasional field experiments have also demonstrated impacts of sublethal infections on reproductive rates (Munger and Karasov, 1991) and susceptibility of wildlife to predation (Hudson *et al.*, 1992). Yet the vast majority of empirical studies consider wildlife pathogens and parasites only if they threaten the health of humans or their domestic animals, and often overlook their natural history. Partly this reflects the priorities of funding agencies; and their concerns are not trivial. More than 70% of emerging (or re-emerging) pathogens of humans are thought to have wild animals as their natural reservoirs (Taylor *et al.*, 2001). Examples include SARS-CoV (severe acute respiratory syndrome coronavirus), avian influenza A (H5N1) virus (bird flu), NIPAH virus, hantavirus and West Nile virus. The economic imperative for controlling zoonoses that affect domestic stock can be very strong. For example, bovine tuberculosis (bTB) in the United Kingdom undoubtedly has a reservoir in wild badger populations, and the direct cost of the disease to agriculture is projected to reach £1 billion by 2011 (Department for Environment, Food and Rural Affairs (DEFRA), 2004).

It has been recognised relatively recently that disease can also pose a serious threat to the survival of endangered wildlife (Lyles and Dobson, 1993; May, 1988; Smith, 1982). This can either be through direct mortality, where losses are greater than the birth rate, or through effects on birth rate, longevity and survival, which suppress the population size to a level that renders it susceptible to extinction by stochastic effects (Table 8.1). Generalist pathogens with a wide host range are particularly problematic, since even virulent species can persist in alternative hosts while driving the rare host to extinction (Begon and Bowers, 1995). Although remaining low on the list of priorities compared with other threats such as habitat loss, efforts are therefore sometimes now made to control infectious diseases for conservation reasons. Examples of recent successes include the control of canine distemper virus in black-footed ferrets (*Mustela nigripes*) (Williams *et al.*, 1988), and rabies in African wild dogs

TABLE 8.1 Examples of spill-over of infection from a common reservoir host that has threatened the viability of an endangered population

Threatened species	Pathogen	Reservoir host	Mechanism	References
Ethiopian Wolf *Canis simensis*	Rabies, CDV	Domestic dogs	Direct mortality	Randall *et al.*, 2004
Black-footed ferret *Mustela nigripes*	CDV	Badgers/coyotes	Direct mortality	Williams *et al.*, 1988
Bighorn sheep *Ovis canadensis*	*Pasteurella*	Domestic sheep	Direct mortality	Jessup *et al.*, 1991
African wild dog *Lycaon pictus*	Rabies	Domestic dogs/jackals	Direct mortality	Gascoyne *et al.*, 1993; Scheepers and Venzke, 1995
Lions *Panthera leo*	CDV	Domestic dogs	Direct mortality	Roelke-Parker *et al.*, 1996
Mountain Gorilla Red squirrels *Sciurus vulgaris*	Measles Squirrel pox	Possibly humans Grey squirrels	Direct mortality Direct mortality	Ferber, 2000 Sainsbury *et al.*, 2000
White-clawed crayfish *Austropotamobius pallipes*	Crayfish plague	Signal crayfish	Direct mortality	Holditch and Reave, 1991
Baikal seal *Phoca sibirica*	CDV	Domestic dogs	Direct mortality	Mamaev *et al.*, 1995
Grey wolf *Canis lupus*	Parvovirus	Domestic dogs	Cub mortality affecting viability of small populations	Mech and Goyal, 1995
Mednyi arctic foxes *Alopex lagopus semenovi*	Otodectic mange	Domestic dogs?	Cub mortality affecting viability of small populations	Goltsman *et al.*, 1996

Note: CDV, canine distemper virus.

(*Lycaon pictus*) (Hofmayer *et al.*, 2004) and Ethiopian wolves (*Canis simensis*) (Haydon *et al.*, 2004).

In Europe alone, there are at least 35 zoonotic parasites and pathogens in wildlife that are known to be important either to public health or the agricultural economy (Artois *et al.*, 2001). For many other infectious agents, such as *Cryptosporidium parvum*, the epidemiological role of wildlife is unknown. Despite the many attempts to control zoonoses in wildlife, the success rate is poor. Typically, measures are adopted as crisis management (usually in the form of culling) following an outbreak, with little understanding of the ecology of species or its relationship to the pathogen. Crisis management also means that proper scientific designs with appropriate controls are often lacking; it is therefore difficult to evaluate the effectiveness of a given intervention. Even where there is a long history of attempts to control a disease through the management of a wildlife reservoir, the results have not been encouraging. For example, efforts over the last 30 years to control bTB in cattle in the United Kingdom by culling of badgers has failed to yield significant benefits, with analyses of the recent randomised controlled trial of badger culling concluding that culling could not contribute meaningfully to future control strategies for bTB (Donnelly *et al.*, 2005). Similarly, the culling of foxes has been discounted as a means of rabies control in western Europe (Blancou *et al.*, 1991). However, success has been achieved through the use of widespread vaccination (administered via bait) (Aubert, 1995). Rabies is currently the only example of a widespread strategy of vaccination being favoured over the control of the host species (Artois *et al.*, 2001).

This review considers the ecology of zoonoses in wildlife and the links between infection in wildlife and humans or livestock. It proposes that a shift to ecologically based control, explicitly considering the natural history of wildlife hosts and their pathogens, is crucial in minimising the risk presented to humans, domestic animals and endangered species from zoonoses. This approach will also yield benefits for the conservation and welfare status of wild animals.

8.2. PATHWAYS LINKING PATHOGENS IN WILDLIFE TO OTHER HOSTS

8.2.1. What data are available and what drives collection efforts?

Notwithstanding the complexities of specific relationships, the probability of a zoonosis being passed from wildlife into another host population, be it humans, domestic stock or an endangered wild species, is always influenced by several key parameters. These are the intensity of infection

in the reservoir hosts; the size, or depending on the case, the density, of the infected host population; the degree and nature of the contact between infected individuals (or infectious particles in the environment such as infected faeces); and the susceptibility of the in-contact animal. (For indirectly transmitted parasites and pathogens, the role of vectors and/or intermediate hosts must also be considered.)

Whether the zoonosis persists after initial invasion is also determined by the new host's population size. A great variety of models has been developed to describe the transmission dynamics of macro- and micro-parasites, taking into account the nuances of particular host and parasite population structures (Diekmann and Heesterbeek, 2000; Heesterbeek and Roberts, 1995; Scott, 1988; Scott and Smith, 1994). Yet empirical research has lagged behind the theoretical advances. The legacy of researchers like Elton and Chitty (Chitty, 1952, 1954; Elton, 1931; Elton *et al.*, 1931, 1935), who sought not only to describe pathogens but to understand their ecological role, has not been sustained. (There are a few notable exceptions, including the long-term studies of small mammals in the north of England (Beldomenico *et al.*, 2008a,b; Feore *et al.*, 1997); rodent reservoirs of hantavirus in the United States (Calisher *et al.*, 2007; Mills *et al.*, 1997) and macroparasite infections in laboratory models (Ehman and Scott, 2004; Scott, 1988; Scott and Anderson, 1984; Scott and Smith, 1987).) This deficiency was noted in a key text in the field in 1995 (Grenfell and Dobson, 1995) and again in the follow-up publication in 2002 (Hudson *et al.*, 2002).

We lack even species lists of parasites and pathogens for most, if not all, wild animals. While pathogens that affect international trade are reported to the World Organization for Animal Health (OIE), and many of these affect wildlife (see Artois *et al.*, 2001) for the lengthy list of those likely to affect wildlife in Europe), there is no agreed systematic programme of surveillance (Kulken *et al.*, 2005). Even where programmes exist, they lack integration with surveillance in humans and domestic animals at both local and international scales. Disease surveillance in wildlife is usually driven by outbreaks in humans or domestic animals (Childs, 2004). Virulent pathogens are, therefore, more likely to be detected than more benign ones (Williamson *et al.*, 1986). Such studies are also, by their nature, not designed to screen for a range of pathogens, so opportunities to investigate the epidemiology and ecology of co-infections are often lost. Systematic surveillance of representative samples of the population is difficult and time consuming. Yet prevalence estimates can be seriously skewed if the only data available are derived from passive surveillance of carcasses. Not only are estimates likely to be too high if they are based on samples of wildlife found dead or sick by the public, but even road kills and game bags are likely to over-represent certain population classes (such as dispersing juvenile male mammals) and animals in compromised health.

Disease-responsive surveillance also offers little information on the frequency with which transfer events are likely to occur. For example, many of the 'spectacular' epidemics derived from bat viruses, such as Hendra virus, Nipah virus, SARS-CoV-like virus, have been observed only a small number of times. We do not know why this should be the case. Is transfer of zoonoses from bats to terrestrial vertebrates generally rare due to a lack of appropriate contact? Or is there regular inter-specific transmission of other viruses but these go undetected because they lack the extreme pathogenicity of Hendra and Nipah viruses to stimulate screening efforts? Pro-active surveillance of wildlife and of apparently healthy human or livestock populations could help answer these questions.

8.2.2. What can surveillance data reveal about geographical structuring and species specificity of a pathogen in wildlife hosts?

Screening for European bat Lyssaviruses in Europe is an exemplary case of research stimulated by public health concerns. The first recorded European bat rabies case was in Hamburg in 1954 and several other cases were identified subsequently (King *et al.*, 2004). Yet surveillance of bats was not really pursued until a woman in Denmark was bitten by a serotine bat (*Eptesicus serotinus*) infected with European bat Lyssavirus 1 (EBLV1). Since then more than 800 rabies-positive bats have been identified across Europe; the vast majority being serotine bats infected with EBLV-1 (Harris *et al.*, 2006). In the United Kingdom, screening efforts were intensified following the death of a man in Scotland from EBLV-2 in 2002, after apparent contact with many bats in the United Kingdom and Europe (Fooks *et al.*, 2003). In contrast with classical rabies (RABV) there is now good evidence that at least some bats (and possibly other animals) can produce neutralising antibodies and survive EBLV infection for at least 6 years (Serra-Cobo *et al.*, 2002; van der Poel *et al.*, 2000), and experimental models suggest that EBLV-2 might be inherently less virulent than EBLV-1 (Vos *et al.*, 2004). EBLV-2 also appears to have a much more restricted geographical range than EBLV-1, and small numbers of positive bats have been identified in the United Kingdom, Switzerland, the Netherlands, Denmark and Germany (Department for Environment, Food and Rural Affairs (DEFRA), 2008; Racey *et al.*, 2004; Vos, 2007; Vos *et al.*, 2007). These cases have all been in the closely related Daubenton's (*Myotis daubentoni*) and pond bats (*M. dasycneme*).

Structuring of EBLVs therefore is apparent from these data both across geographical areas and across species. The serotine bat occurs over most of Europe, extending north to 55° latitude (England south of the Wash estuary, Denmark and southern Sweden); Daubenton's bats are common

across Europe; and the pond bat is present in a wide band across central and eastern Europe (between 48° and 60° latitude; absent from the United Kingdom (Schober and Grimmberger, 1997)). Yet neither EBLV-1 nor EBLV-2 appears throughout their hosts' ranges. Some suggest that in the case of EBLV-1, this may be because long-distance travel is uncommon in serotines, the primary host (Vos *et al.*, 2007). Yet the species is widely distributed, and it is unlikely that there are gaps between populations that could not be travelled with relative ease; dispersing movements of up to 300 km have also been recorded (Hutterer *et al.*, 2005). Interestingly, EBLV-2 also appears to have a patchy distribution, despite its host species, at least in continental Europe, being migratory over long distances (Vos, 2007). Whether the geographical distribution is, in reality, less patchy than it currently appears requires co-ordinated surveillance effort and a willingness by statutory authorities to publish test results even if they are negative. It is clear that active surveillance (systematic screening of bats in the wild) has been undertaken in a few countries only, and passive surveillance (submission of dead bats by members of the public for screening) has involved few, if any, animals in a number of European countries, including Portugal, Ireland, Greece, the Czech Republic and Slovakia (Racey *et al.*, 2004).

A range of European bats, most of which are common and widespread, has been identified as having active EBLV infections in addition to the key hosts (Table 8.2). It is striking then that the vast majority of reported cases come from just three species. Undoubtedly, the numbers of bats of each species submitted by the public does not match their abundance in the wild, but is influenced by the closeness of their contact with humans (and their cats, which are a major cause of bat mortality). For example, few woodland specialists have been submitted, whereas bats that frequently roost in houses, particularly pipistrelles, long-eared bats and possibly serotines, are over-represented (Harris *et al.*, 2006). Even active surveillance does not attempt comprehensive surveys of all species in proportion to their abundance: instead, it focuses on the three species already identified as being important sources of EBLVs, potentially failing to estimate properly the prevalence in others.

Despite these limitations, the data clearly suggest that species partitioning occurs. The common pipistrelle bat is known to be susceptible to experimental infection with EBLV-1 (Kuzmin and Botvinkin, 1996). Yet none of the more than 10,000 pipistrelles (*P. pipistrellus* and also *P. pygmaeus*, which is cryptic with *P. pipistrellus*) surveyed in the Netherlands, France and the United Kingdom (Harris *et al.*, 2006; Picard-Meyer *et al.*, 2006; van der Poel *et al.*, 2005) has proved positive for the virus. Whether structuring across bat species driven by differing immunoresponsiveness to particular EBLV types, by a lack of transmission opportunities or by other mechanisms, is unclear.

TABLE 8.2 Bats in Europe identified as actively infected with EBLV-1 or -2 in Europe[a]

Species	Common name	Test	Distribution of bats in Europe[b]
Eptesicus serotinus	Serotine	FAT	Throughout Europe, except the far north (in United Kingdom restricted to southern England)
Nyctalus noctula	Noctule	FAT	Throughout Europe except the far north (in United Kingdom absent from Scotland)
Myotis daubentoni	Daubenton's	FAT	Throughout Europe
M. dasycneme	Pond	FAT	Throughout Europe except the far north (absent from United Kingdom)
M. myotis	Greater mouse-eared	FAT	Central and southern Europe
Pipistrellus nathusii	Nathusius's pipistrelle	FAT	Central, southern and eastern Europe. Records now also regularly occurring in United Kingdom, but population status uncertain
Pipistrellus pipistrellus[c]	Common pipistrelle	FAT	Throughout Europe
Plecotus auritus	Brown long-eared	FAT	Throughout most of Europe
Barbastellus barbastellus	Barbastelle	RT-PCR[d]	From southern England to the Caucasus. Absent from parts of southern Europe
M. nattereri	Natterer's	RT-PCR	Throughout most of Europe
Miniopterus schreibersii	Schreiber's	RT-PCR	Southern and eastern Europe
Rhinolophus ferrumequinum	Greater horseshoe	RT-PCR	Central and southern Europe, parts of northern Europe (including south-west England and south Wales)

[a] Data derived from citations (Vos *et al.*, 2007)*
[b] Schober and Grimmberger, 1997
[c] *P. pygmaeus* rarely identified in testing records, as the species is cryptic with *P. pipistrellus*, the two species only being distinguished recently
[d] This study used RT-PCR with retro-pharyngeal swabs (Echevarria *et al.*, 2001). The technique is considered more sensitive than conventional FAT.
Notes: FAT, fluorescent antibody test; RT-PCR, reverse-transcriptase polymerase chain reaction.

Multi-species summer, and particularly hibernation, roosts are known, though the amount of inter-specific direct contact appears to vary by season and species. For example, bats in houses and trees tend to use single-species roosts, even if more than one species is present at the site (Park *et al.*, 1996). There may be more potential for inter-specific contact at key underground sites used by bats. In a survey of more than 76,000 bats of 13 different species roosting in caves in Turkey, it was noted that multi-species clusters frequently occurred in the post-hibernation season, but not during hibernation; and the horseshoe bats (*Rhinolophus* spp.) only ever formed single-species clusters (Furman and Özgül, 2004). Many bat species also use swarming sites—enclosed areas often in and around caves—for display purposes. At these sites, hundreds or thousands of bats of mixed species congregate (Glover and Altringham, 2008; Parsons *et al.*, 2003). The amount of contact, for example, via urine or aerosol droplets, between species at these events is unknown. More field research is needed to investigate the opportunities for disease transmission across bat species, and across geographical barriers.

Interestingly, in the United Kingdom, only a single case of exposure to EBLV-1 has been found (the test was able to detect exposure rather than live virus), whereas in other European countries with EBLVs, the apparently more pathogenic EBLV-1 is more common (DEFRA, 2008; Racey *et al.*, 2004; Vos, 2007). To date, seven Daubenton' bats (*Myotis daubentoni*) in the United Kingdom have been found to have EBLV-2 infection (DEFRA, 2008), the latest case being diagnosed in May 2008. It is notable that although a low prevalence (around 2%) of seropositivity was detected during active surveillance of Daubenton's bats in Scotland, live virus was not isolated from any of them (Brookes *et al.*, 2005). Similar results were found in an active-surveillance study in Spain, which found that up to 60% of individuals in some colonies were seropositive for EBLV-1, but the prevalence of active infection was less than 1.1% (Serra-Cobo *et al.*, 2002). It is currently difficult to interpret these results, but the vertical transmission of antibodies, as well as acquired immunity, is a possibility.

While it is clear that EBLV-1 and EBLV-2 can cause deaths in unvaccinated humans, whether natural immune responses and cryptic recovery (i.e., without the virus having invaded the central nervous system and become symptomatic) are possible remains unknown. It is unfortunate that there was been no serological testing of bat workers in the United Kingdom to establish the natural prevalence of neutralising antibodies to EBLVs prior to 2002. Since that date, following the fatality in Scotland from EBLV-2, it has been officially recommended that bat handlers be vaccinated against rabies. The take-up rate of vaccination has been very high. This understandable management of the public-health crisis means that it is now not possible to gather information that would have helped

indicate the pathogenicity of EBLV-2 to humans, and also whether exposure was more widespread than the single fatal case. It certainly appears that despite other species being susceptible in experiments, natural spill-over into other non-bat hosts to produce clinical symptoms is rare, with the only known case for EBLV-1 being a single stone marten (Muller *et al.*, 2004). There are no reports of spill-over for EBLV-2.

8.2.3. Stages of infection and intensity of infection

The apparently simple task of establishing the prevalence of a pathogen in wild animals can be fraught with difficulty. Even assuming that a reasonably random cohort can be sampled, there is usually no opportunity to repeat 'live-tests' in cases of diagnostic uncertainty. Establishing values for other key parameters is equally problematic. Fundamental data on the sex- and age-distributions of infection are often not recorded. Sometimes this is because the surveys (particularly for 'crisis management') were not designed with research in mind. Sometimes it is practically difficult for the data to be acquired. Bats, for example, can live more than 30 years, yet it is impossible in the absence of long-term banding studies on the particular population being surveyed, to judge the age of animals with much greater accuracy than 'juvenile', 'young of the year', and 'adult'. Weight is frequently used as a surrogate for age or maturity, particularly in studies of rodents, but there can be difficulties in distinguishing age from dominance effects, since both are correlated with body mass. The size of the population (or its density) is also often estimated with huge margins of error, as surveyors simply lack the time to undertake detailed ecological studies in addition to collecting clinical samples.

Distinguishing between different burdens of infection (particularly for macro-parasites) and stages of infection (particularly for micro-parasites) is frequently overlooked. This makes it difficult to use the data to parameterise epidemiological models. For example, animals infected with bTB but in which the bacilli are encysted present no risk of transmission at that particular time point, yet these groups are often combined when data from post-mortem examinations are used. The fact that the disease may reactivate at some future time (measured by the overall prevalence) is not relevant to the calculation of the basic reproductive rate of the disease R_0.

By conducting large-scale surveys of representative populations of wildlife on British farms, workers were able to build deterministic models to investigate the likelihood of the disease persisting in each host species. Initially they assumed that no between-species transmission was present. Using the prevalence of infectious individuals, together with field data on population structure and density derived from the same sites, they computed the basic reproductive number R_0 for each of the species. The analyses showed that even when the maximum likely prevalence was

assumed (based on the upper 95% confidence limit), the R_0 (the basic reproductive rate of the disease) ranged from just 1.003 in wood mice to 1.05 in rats. (The lower confidence intervals for prevalence always gave R_0 values that were <1.0; Mathews *et al.*, 2006b.) It is therefore unlikely that the disease would persist within single-host systems in the wild: the animals are unable to pass on the infection to their conspecifics at a rate high enough to maintain the disease. The findings are robust to under-diagnosis of infection: to affect the R_0 materially, the prevalences would need to be have been underestimated very substantially. If, instead of single-host models, we assumed multiple-host systems, then higher prevalences should have been observed in the field than those recorded. Alternatively, to achieve the prevalences seen in reality, the within-species transmission rate would have to be even lower than the very low value calculated. They have therefore been able to conclude that multi-species transmission of bTB within farmland wildlife communities appears unlikely.

Perhaps the best example of long-term epidemiological studies in wildlife leading to epidemiological models of value to human health comes from studies of hantavirus infection in the United States. Large-scale studies of several thousand rodents were conducted by four separate research teams, but were co-ordinated by common methodologies (Calisher *et al.*, 2007; Easterbrook *et al.*, 2007b; Glass *et al.*, 2007; Mills *et al.*, 1997). Using long-term datasets, with repeated trapping at set grids, the teams were able to explore key components of the transmission pathway. Seropositivity was higher in males and in heavier animals, suggesting horizontal transmission among adult males. Decreasing prevalence with age among the youngest deer mice suggests that infected dams confer passive immunity to pups. In the main host of Sin Nombre virus, the deer mouse (*Peromyscus maniculatus*), gender, age, wounding, season and local relative population densities were linked with the period prevalence of antibody (used as a marker of infection). Nevertheless, antibody prevalence and some of the risk factors associated with antibody prevalence, such as relative population density, gender bias and prevalence of wounding, varied significantly among sites and even between nearby trapping arrays at a single site. This suggests that local micro-site-specific differences play an important role in determining relative risk of infection in rodents and, consequently, in humans. These data are now being used in spatially explicit models of the risk of human disease outbreaks (Eisen *et al.*, 2007).

8.2.4. Contact rates and host population size

As described for bat lyssaviruses, the contact rates between infectious and susceptible individuals (or a vector and a susceptible) is a critical step in the transmission pathway. Yet compared with the effort that goes into

improving, for example, the accuracy of a diagnostic test, very little attention is paid to measuring it in the field. This failure may offer some explanation for the difficulties faced in attempted disease control programmes.

For example, disease is a primary threat to the survival of the critically endangered Ethiopian wolf (*Canis simensis*). Since the early 1990s, outbreaks of rabies and canine distemper virus (CDV) have had significant impacts on wolf population dynamics (Marino *et al.*, 2006; Randall *et al.*, 2004, 2006). These diseases are maintained in local domestic dog populations, and a programme of dog vaccination was therefore introduced in 1996, with the aim of reducing the population of susceptible dogs and hence the risk of transmission to wolves. Attempts were made to achieve coverage of more than 70% of susceptible dogs during annual vaccination campaigns. This was not an easy task since dogs in Ethiopia are used for guarding cattle and are not tame. Rabies vaccines have high efficacy, and in theory, this level of coverage should prevent rabies outbreaks 95% of the time (Coleman and Dye, 1996; World Health Organization, 2004). Over 30,000 vaccinations have taken place, and at least initially, the number of rabies cases in dogs declined (Randall *et al.*, 2006). Nevertheless, a rabies outbreak occurred in wolves in 2003, and could be linked with more than 35 sympatric dogs with clinical symptoms consistent with rabies (Randall *et al.*, 2004). Mathews has, therefore, been analysing the reasons for the apparent failure of the vaccination strategy, focusing on the population dynamics of the domestic dogs, using data collected by the Ethiopian Wolf Conservation Programme. The key factor appears to be the growth of the dog population, which, as in other African countries, is keeping pace with, or even outstripping, human populations (Cleaveland *et al.*, 2000; Rhodes *et al.*, 1998). Eighty-six percent of all households owned dogs, rising to 93.5% in rural areas. Virtually nothing is known about the true contact rate between domestic dogs and Ethopian wolves. It is clear that interactions do occur as wolf–dog hybrids are seen. We might speculate that diseased dogs, and aggressive dogs that are difficult to vaccinate, might be even more likely to interact with wolves than would healthy ones.

Some data are available on the demography of the dogs surrounding the Bale Mountains National Park—one of the strongholds of the remaining Ethiopian wolf population—as a result of a questionnaire survey administered by the Ethiopian Wolf Conservation Programme. The rate of increase in the dog population size appears to be around 5% per annum, and the turnover rate is also high. This creates a constant influx of new susceptibles into the dog population. It is difficult to keep pace with these, given the financial and logistical constraints on the numbers of visits veterinarians can make to each village. There also appears to be some geographical clustering of vaccination effort, and the implications of

pockets of unvaccinated dogs on the probability of rabies transmission to wolves is currently being explored.

The vast majority of efforts to control zoonoses in wildlife hosts, rather than in domestic animals, rely on culling strategies. In simple terms, the idea is to depress the population of the reservoir host to a level at which the disease can no longer be sustained, because the density of infected and susceptible hosts is too low. Few of these culling programmes have systematically examined either the total population size or the level of population reduction likely to be required to achieve the desired end-point. Even where this has been done, it can be difficult practically, as with vaccination, to achieve the level of coverage desired.

The strategy to control bTB in badgers and cattle in the United Kingdom has had the culling of badgers as its cornerstone for more than 30 years. When it became evident that gradual badger culling was having little or no impact on the incidence of the disease in cattle, a formal review of the programme was introduced, culminating in a large-scale randomised trial of badger culling. This has demonstrated that there is no clear-cut reduction in bTB in cattle. On the contrary, whereas the incidence of bTB in farms at the centre of 100 km^2 badger culling zones fell by around 19%, the incidence in farms up to 2 km away from the borders of these zones increased by around 29% (Donnelly, 2005). Similar results were found in comparable areas where clusters of badgers were removed reactively, following nearby outbreaks of bTB in cattle (Donnelly *et al.*, 2003).

At least part of the explanation for the failure to achieve effective bTB control is likely to be the alteration in contact rates between infected and susceptible badgers, and also between infected badgers and cattle, as a result of the culling. There has been only one detailed study of *M. bovis* epizootiology in undisturbed badgers (culling having been suspended at the site, Woodchester Park in Gloucestershire, England in 1978; Delahay *et al.*, 2000). This study showed that bTB does not spread rapidly at high incidence through badger populations, but rather is distributed patchily among a minority of individuals. Social groups are relatively stable, and long-term dispersal movements are uncommon, though shorter movements do occur more regularly (Rogers *et al.*, 1998; Vicente *et al.*, 2007). There is a correlation between rates of inter-group movement and the incidence of new infections (Rogers *et al.*, 1998; Vicente *et al.*, 2007). While spatial clusters of infection exist, there is no strong synchrony between neighbouring groups, suggesting that there is only limited transmission between adjacent social groups (Delahay *et al.*, 2000). Both individuals and groups are more likely to be incident cases where the social group was diminishing in size, although there is no apparent relationship with group size itself, suggesting that it is the change in group size, and possibly the associated social dynamics, that influences disease risk (Vicente *et al.*, 2007).

Badger culling operations have clear impacts on the behavioural ecology of the survivors. Woodroffe *et al.* (2006) found that badger social group ranges increased among survivors within reactive and pro-active culling areas and along the perimeters of pro-active culling areas. Their finding, at a large scale, accords closely with the observations of Macdonald *et al.* (2006) of individual and group behaviour in two zones of badger removal in England, as well as those of O'Corry-Crowe *et al.* (1996) in Ireland. In all cases, the spatial organisation of social groups was considerably altered following the culls, with a large increase in the extent of overlaps between social groups (e.g., Fig. 8.1). The numbers of ranges with which each group overlapped also increased. There was a rather chaotic alteration in population densities (e.g., Fig. 8.2). In the examples shown, culling was conducted in 1995, largely targeting areas of highest badger density (in effect, the largest social groups). One year later badger density was, unsurprisingly, lower in the culled areas, whereas there had been some increases elsewhere. In 1997, although the population as a whole had not grown, the density remained low, or even fell further, in two removal areas, but increased elsewhere. By 1998 and 1999, the distribution of badgers in the study area was radically different from that at the outset, with some previously high density, but culled, areas remaining depauperate (Macdonald *et al.*, 2006). Thus, while the population density recovered as a whole, the badgers built up in a different place. This sort of radical redistribution has not been reported in undisturbed populations. The changes have not only implications for absolute contact rates, but also the nature of contacts. For example, bite-wounds—an important route of bTB transmission—were more common in the Macdonald *et al.* (2006) study following social perturbation.

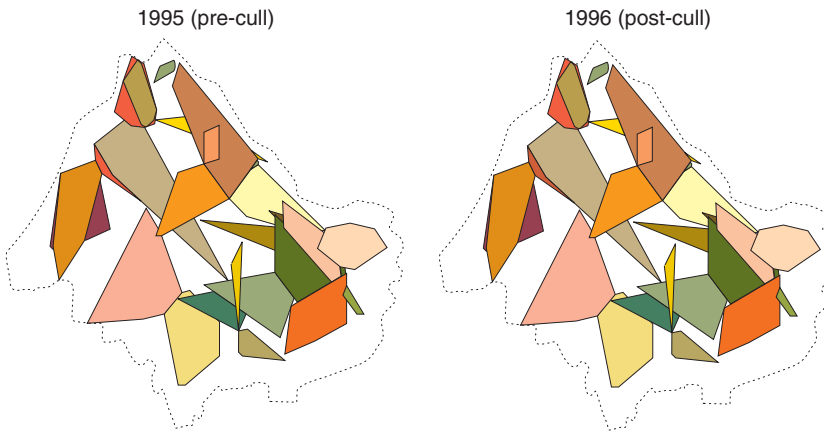

FIGURE 8.1 Badger social territories before and after the selective removal of social groups following bTB incidents in local farms.

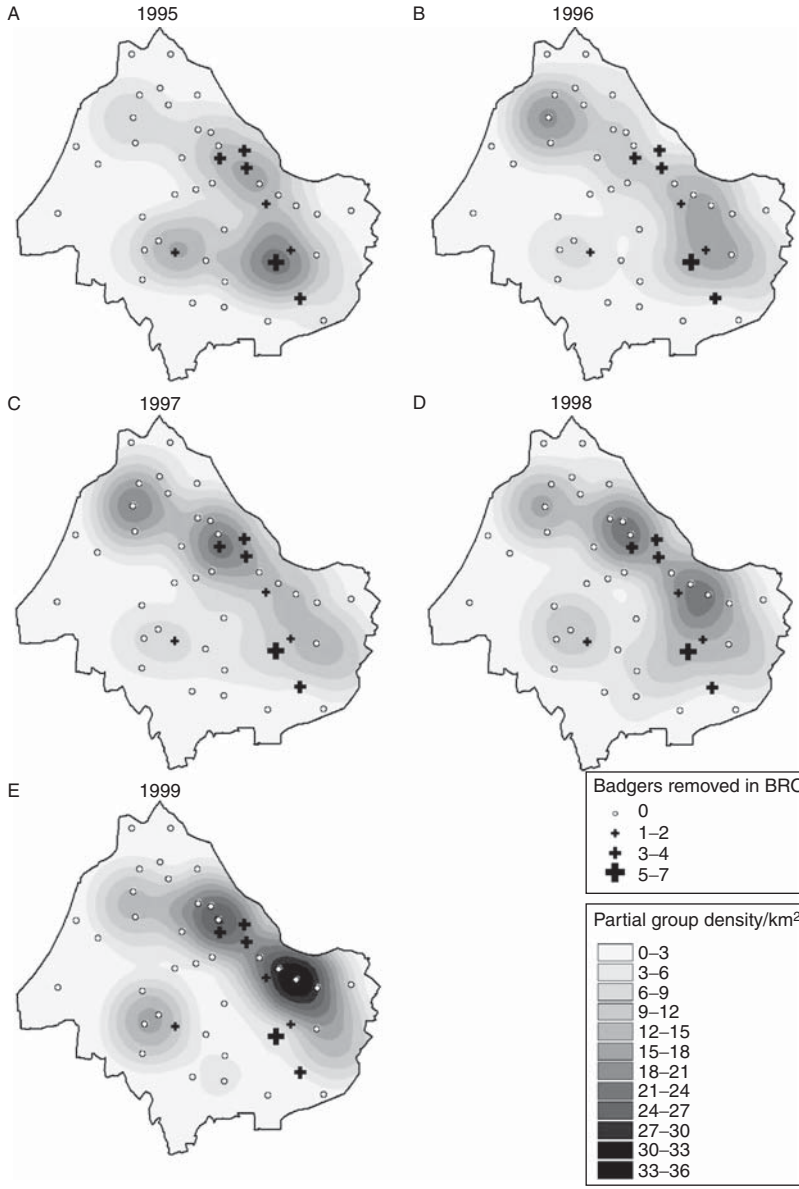

FIGURE 8.2 Changes in badger population density following badger removals (BROs) Source: Macdonald *et al.*, 2006.

Social structure also plays an important role in hantavirus transmission. In deer mice, both wounding and Sin Nombre virus antibody prevalence increased with mass. Although it occurred in both sexes, the

increase was much more pronounced in males. Wounding was more frequent in adult males than in adult females, and adults had more wounds than juveniles. The highest rate of infection was seen in individuals with the most wounds. Similarly, in rats (*Rattus norvegicus*) hantavirus infection (Seoul virus) was associated with both wounding and elevated testosterone levels (Easterbrook and Klein, 2008; Easterbrook *et al.*, 2007a). It is therefore evident that changes to social structure—for example, by the removal of a dominant male—could have important implications for the epidemiology of a disease.

8.3. INTEGRATING DISEASE AND WILDLIFE ECOLOGY INTO CONTROL STRATEGIES

Rather than attempt to control disease by vaccination or culling, an alternative approach is to understand the factors leading to disease outbreaks in the first place and to manage these (Dobson, 2005). Habitat changes that lead to alterations in population structure or migratory patterns, for example, are likely to affect the risk of zoonotic disease transmission (Dobson and May, 1986). The effect of habitat fragmentation on disease processes has rarely been investigated, but it has recently been shown that *Trypanosoma cruzi* infection rates are higher in fragmented than continuous Atlantic forest (Vaz *et al.*, 2007); and the risk of Lyme disease in New York is also apparently increased by fragmentation (Allan *et al.*, 2003). Interestingly, the division of endangered Ethiopian wolf population into small sub-populations, joined by habitat corridors, has been shown to allow rabies control to be achieved using a low-coverage vaccination strategy (Haydon *et al.*, 2004). The strategy operates by eliminating the largest outbreaks of disease, and so enhances meta-population persistence, rather than by the conventional objective of reducing the reproductive number of the disease to less than one (Haydon *et al.*, 2004). Human activities that artificially increase, rather than decrease, animal densities also influence disease processes. These increases can be the result of losses of absolute habitat area, or from the provision of supplementary food or water. In the United States, the practice of supplementary feeding of house finches and white-tailed deer has lead to an increase in the incidence of mycoplasmal diseases and bTB, respectively (Hartup *et al.*, 1998; Schmitt *et al.*, 1997), presumably because of greater opportunities for disease transmission, and possibly also immunosuppressive effects of aggression at the feeding sites.

bTB has also been an intractable problem in the British cattle herd, with the incidence rising inexorably since 1979. With a cull of badgers recently being ruled out, somewhat controversially, as offering no meaningful contribution to the long-term control of the disease (Donnelly *et al.*, 2005), it is worth asking whether consideration of the ecology of the badgers and

cattle might help generate workable solutions. Over the past 30 years, along with the increase of bTB, there has also been an increase in badger densities, and it is likely that this contributes at least in part to the disease in cattle. So why have badger populations risen? Might the answer lie in changes in land use? Macdonald and Newman (2006) speculate on a possible role for climate change, with milder winters and hence greater earthworm availability improving survival rates.

Changes could also have occurred in the susceptibility of badgers to bTB and/or of cattle to bTB. For example, the average milk production of a dairy cow rose from 3,750 l in 1970 to 5,395 l in 1995 (Farm Animal Welfare Council, 1997), possibly to the detriment of the animal's immune status. Similarly, stress resulting from cull-associated social perturbations, or from other changes to habitat, food availability or population density, may have influenced the innate immune response of badgers. Little is known of the physiological responses of free-living wild mammals to poor environmental quality or other potential stressors. An argument has been made for polychlorinated biphenyls (PCBs) and other pollutants contributing to phocine distemper outbreaks (Ross *et al.*, 1996, 2000), but this is has been questioned (O'hea, 2000). While the role of toxicants is not clear, recent work indicates that high population densities in wild field voles is associated with compromise in haematological and immunological indices. Poor body condition appeared to affect the inflammatory response (as indicated by lower neutrophil and monocyte peaks) and lower immunological investment (as indicated by lower lymphocyte counts (Beldomenico *et al.*, 2008a,b).

I have found, with co-worker Jon Blount, preliminary evidence of increased oxidative stress (measured by serum malondialdehyde concentration) among non-infected badgers from farms with recent bTB in cattle, compared with those at sites free of bTB (Fig. 8.3). There is also a

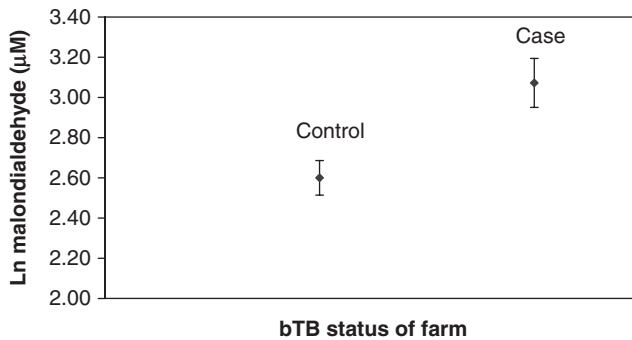

FIGURE 8.3 Mean levels of oxidative damage (+/− 1 standard error of the mean (SE)) in the serum of 19 badgers by farm bTB status. The difference between the two groups was significant, $t = -2.896$, $df = 16$, $p = 0.011$.

considerable literature from farm (Moberg and Mench, 2000), laboratory (Galloway and Handy, 2003), and free-living aquatic animals (Liney *et al.*, 2006) showing that environment has a strong impact on stress responses, and that these can lead to pathological and pre-pathological alterations in immune function and overall health status.

While it might be difficult to intervene directly to reduce the causes of stress in animals, ecologically based interventions that reduce both disease susceptibility and the opportunities for transmission may be possible. For example, in a study of 120 British farms it has been shown that habitat management and cattle herd size were strongly associated with the risk of bTB in dairy cattle (Mathews *et al.*, 2006a). Reduced risk of bTB was associated with the management of farmland in ways favourable to wildlife, including greater hedgerow availability, a lack of gaps in hedgerows, increasing hedgerow width and the presence of ungrazed wildlife strips adjacent to hedgerows. All of these measures are encouraged by recent European Common Agricultural Policy reforms (2005). Broadly, habitat could influence cattle contact rates or be associated with agricultural management practices in ways relevant to bTB transmission (such as reduced herd size). Favourable habitat may lower the susceptibility of badgers to bTB or reduce the number of inter-group excursions; alternatively, cattle on hedgerow-rich farms may be at reduced risk of ingesting contaminated soil.

Taking for simplicity just one of the parameters contributing to the effects—the total length of hedgerow—an increase of 1 km/100 ha was associated with a decrease in the odds of bTB by about 12.5% (95% confidence interval 0.3% increase to 26.3% decrease) in univariate analysis. In absolute terms, this equates to the annual risk of bTB changing from the current rate of 9.2% (2,152 confirmed incidents in 23,471 herds in 2004) to 8.1% (1,901 incidents) for herds in the west of England if a policy of moderate hedgerow density increase were adopted. This would mean a reduction of 251 infected herds per year. By comparison, systematic badger culling appears able to reduce the odds of bTB by a maximum of about 19% and may even increase the prevalence in neighbouring areas (Donnelly *et al.*, 2005).

Change in land use has also been linked to the emergence of two henipaviruses, Nipah virus and Hendra virus in the 1990s, and land use management may therefore offer part of the solution. Both viruses appear to be asymptomatic in their natural hosts, fruit bats (genus *Pteropus*). They are amplified in domestic animals, pigs and horses, respectively, where they cause mortality, and can then be passed on to humans (Chua *et al.*, 2000; Halpin *et al.*, 2000). The closeness of RNA sequence match between *Pteropus* sp., livestock and human isolates of each virus suggests that a sudden change in virulence is a less likely explanation of their rapid emergence into domestic animals and humans than is the ecological

change that have affected the habitat of their natural hosts. Many flying fox species are in decline, with roosting and feeding sites being deforested, and converted to agricultural or urban use. A number of hypotheses have been proposed to explain exactly how Nipah virus emerged (see Breed *et al.*, 2006), all of which involve the establishment of piggeries in previously forested regions still used by fruit bats (Chua *et al.*, 2002). Increased contact rates are also the likely explanation for the emergence of Hendra virus in Australia, with many *Pteropus* populations having relocated into urban areas (Hall and Richards, 2000). With no vaccine available, and *Pteropus* in need of conservation, ecologically based strategies to limit contact rates between bats and livestock offer the best prospects of controlling the disease (Field and Mackenzie, 2004).

8.4. CONCLUSION

Managing the risks from zoonoses to the health of humans and domestic animals is complex. It is also fundamentally important: virtually all emerging infectious diseases have originated in wildlife. Superficially, the simplest method of control is via a reduction in reservoir host-disease prevalence, this being achieved by culls of host populations. However, effective reductions in population densities can be difficult to achieve in practice and may be undesirable where the target is of conservation concern. For example, most bat species are threatened, and yet they appear to be particularly important sources of emerging viruses (Calisher *et al.*, 2006; Dobson, 2005); and despite not being endangered, badgers in the United Kingdom are legally protected. An alternative, and possibly complementary, strategy is to manage the ecological conditions leading to disease spill-overs. This will not only benefit the health of humans and their domestic stock, but must surely also lead to benefits for the conservation and welfare of wild animals.

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CHAPTER 9

Understanding the Interaction Between an Obligate Hyperparasitic Bacterium, Pasteuria penetrans and its Obligate Plant-Parasitic Nematode Host, *Meloidogyne* spp.

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Contents

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Abstract

Pasteuria penetrans is an endospore-forming bacterium, which is a hyperparasite of root-knot nematodes *Meloidogyne* spp. that are economically important pests of a wide range of crops. The life cycle of the bacterium and nematode are described with emphasis on the bacterium's potential as a biocontrol agent. Two aspects that currently prohibit the commercial development of the bacterium as a biocontrol agent are the inability to culture it outside its host and its host specificity. Vegetative growth of the bacterium is possible *in vitro*; however, getting the vegetative stages of the bacterium to enter sporogenesis has been problematic. Insights from genomic survey sequences regarding the role of cation concentration and the phosphorylation of Spo0F have proved useful in inducing vegetative bacteria to sporulate. Similarly, genomic data have also proved useful in understanding the attachment of endospores to the cuticle of infective nematode juveniles, and a Velcro-like model of spore attachment is proposed that involves collagen-like fibres on the surface of the endospore interacting with mucins on the nematode cuticle. Ecological studies of the interactions between *Daphnia* and *Pasteuria ramosa* are examined and similarities are drawn between the co-evolution of virulence in the *Daphnia* system and that of plant-parasitic nematodes.

9.1. INTRODUCTION

Over the course of evolution, natural selection has produced marvellous adaptations in which organisms have co-evolved through exquisitely subtle interactions. These adaptations can be observed and studied at different organisational levels that range from the biochemical and molecular, through the cellular and organismic, to the population and ecosystem. Each of these levels of organisation form a part of a nested hierarchy, or holarchy, each of which is complex and necessarily punctuated by a

certain degree of stratified stability if the holarchical structure is not going to collapse to extinction (Bronowski, 1977). Because of the subtle interactions that occur between a host and its parasite, they form a particularly interesting model on which to take a holistic approach and build a coherent understanding of the interactions that integrate the various organisational levels. To survive over evolutionary time, each of these organisational levels within a host–parasitic interaction is complex and has to remain creatively dynamic. This manuscript aims to describe the interactions between the obligate bacterial parasite *Pasteuria penetrans* and its obligate plant-parasitic nematode host, *Meloidogyne incognita*, and integrate our understanding from the biochemical and molecular through to the population and ecosystem level. This will be discussed from the perspective of a scientist involved in developing *Pasteuria* as a biological control agent to control plant-parasitic nematodes.

9.2. PLANT-PARASITIC NEMATODES, BIOLOGICAL CONTROL AND *PASTEURIA PENETRANS*

9.2.1. Nematodes as crop pests

Nematodes are the most abundant metazoans. The group as a whole is cosmopolitan and they are found in most environments. They can be broadly classified into animal parasites, plant parasites and free-living forms. *Caenorhabditis elegans*, a bacterial feeding, free-living nematode, is arguably the most well known and intensively studied nematode and was the first animal to have its genome completely sequenced (Herman, 2004). The plant-parasitic nematodes have been further sub-divided into five groups according to their habitat: migratory ectoparasites, migratory endoparasites, sedentary semi-endoparasites, sedentary endoparasites and above-ground parasites (Winslow, 1960). Plant-parasitic nematodes form part of the community of soil-dwelling nematodes (Yeates *et al*., 1993), and are not of monophyletic origin as their ability to parasitise plants is thought to have occurred on more than one occasion (Baldwin, *et al*., 2004; Blaxter *et al*., 1998; Holterman *et al*., 2006). The most economically important nematodes in agriculture are the sedentary endoparasites such as the root-knot nematodes, *Meloidogyne* spp., and the cyst nematodes, *Heterodera* spp. These nematodes, which affect the majority of arable and vegetable crops, are likely to become increasingly important pests in the context of climate change as they disrupt the ability of plant roots to take up water and nutrients from the soil. Historically, agriculture has developed a range of methods to control these pests that range from cultural methods, involving techniques such as crop rotation and solarisation, to the use of resistant varieties and chemical pesticides. Although chemical pesticides have proved useful in protecting crops from plant-parasitic nematodes, and are

likely to remain important into the foreseeable future, they are among some of the most toxic compounds used in agriculture and alternative approaches are being sought (Rich *et al.*, 2004).

The use of microbial enemies to control plant-parasitic nematodes has a long history (Stirling, 1991) but the concept of a soil being suppressive to nematode pests only really became a focus of intensive study since the latter part of the last century and the problem of cereal cyst nematode. It has long been known that cereals such as wheat and barley are susceptible to the cereal cyst nematode, *Heterodera avenae*, and that this nematode reduces yields considerably. However, during intensive cropping, when it might be expected that nematode populations would devastate yields, it has been recognised that after 4–5 years of continuous cereal cropping the soil becomes suppressive and the nematode population declines (Gair *et al.*, 1969). Intensive study, over the last 40 years has shown that nematode suppressiveness is related to a number of microbial parasites that are present in the rhizosphere and that key organisms could possibly be exploited to develop into a method for the control of nematode pests (Kerry, 2000). One such group of bacteria, the *Pasteuria* group, has potential to be developed into biological control agents (Stirling, 1991).

9.2.2. *Pasteuria penetrans* as a hyperparasite

The most studied of these bacteria is *Pasteuria penetrans*, a Gram-positive hyperparasitic bacterium of root-knot nematodes, *Meloidogyne* spp. The *Pasteuria* group of bacteria are endospore forming and are hyperparasites of plant-parasitic nematodes and water fleas (*Daphnia* spp.; Cladocera: Anomopoda). The fact that they are parasites of such two diverse groups of invertebrates, the Nematoda and the Anomopoda, suggests that there may be other undiscovered *Pasteuria* spp. that infect other known and unknown invertebrates. The detailed taxonomy of this group of bacteria remains unclear but the bacterium is a member of the *Bacillus–Clostridium* clade (Charles *et al.*, 2005; Preston *et al.*, 2003). Most studies have focused on two species of *Pasteuria*, *Pasteuria ramosa* that infect *Daphnia* spp. (Ebert, 2005; Ebert *et al.*, 1996) and *Pasteuria penetrans* that infect plant-parasitic nematodes, including *Meloidogyne* spp. All the economically important genera of plant-parasitic nematodes are parasitised by a *Pasteuria* species (Chen and Dickson, 1998), and to date, five species of *Pasteuria* have been described that differ in their host ranges and pathogenicity on plant-parasitic nematodes: (1) *P. penetrans* is parasitic on *Meloidogyne* spp. (Sayre and Starr, 1985); (2) *P. thornei* parasitises *Pratylenchus brachyurus* (Sayre *et al.*, 1988); (3) *P. nishizawae* parasitises cyst nematodes (Sayre *et al.*, 1991); (4) *Pasteuria usgae* is parasitic on the sting nematode *Belonolaimus longicaudatus* (Giblin-Davis *et al.*, 2003) and (5) *P. hartismeri* is parasitic on *M. ardenensis* (Bishop *et al.*, 2008).

Recently, variable-number tandem repeats (VNTRs) have been used as molecular markers for looking at biotypes (Mouton and Ebert, 2008; Mouton *et al*., 2007) and these will undoubtedly be useful for characterising *Pasteuria* populations parasitic on nematodes. As *P. penetrans* is a hyperparasite of root-knot nematodes, which account for 75% of crop losses due to nematodes, it has potential to be developed into a biological control agent (Stirling 1991) and the majority of studies have focused on the root-knot nematode–*P. penetrans* interaction.

9.2.3. The life cycle of *Pasteuria penetrans* in relationship to root-knot nematodes

Pasteuria penetrans is a member of the endospore forming group of Gram-positive bacteria and the initial stage of the infection process is when infective second-stage juvenile nematodes, migrating through the soil towards plant roots come into contact with endospores that lie dormant in the soil (Fig. 9.1A). Endospores are highly robust and can remain viable for many years (Giannakou *et al*., 1997). The endospores adhere to any part of the infective juvenile cuticle; although there is a slight preference for the anterior region (Davies, unpublished data). The numbers of endospores adhering to a particular juvenile can range from one to around 20 in field soils; however, in standardised attachment assays well over 100 spores per nematode have been observed in some instances. Infective juveniles encumbered with a large number of spores (>15) are less mobile and this reduces their ability to infect plant roots (Davies *et al*., 1988, 1991). Germination of the endospore, which involves a germination peg that breaches the juvenile cuticle (Fig. 9.1B), takes place in the period between the infective juvenile entering the plant root and established a feeding site and before the moult to a third-stage juvenile. However, in some other nematode species, such as *Heterodera avenae*, the endospore can germinate before the juvenile has entered the root (Davies *et al*., 1990) and this has been occasionally observed in root-knot nematodes (Davies unpublished). Recent cryo scanning microscopy observations on developing females infected with *P. penetrans* have revealed that following germination rhizoid structures grow out from the site of infection throughout the pseudoceolomic cavity (Fig. 9.1C) and granular masses of rod shaped Bacilli can be seen (Fig. 9.1D). Similar rod-shaped Bacilli have been observed growing in *in vitro* cultures of *P. penetrans* (Hewlett *et al*., 2004) and are likely to be the exponential growth phase of the bacterium. Electron microscopy has also revealed that some of these rod-shaped bacteria found in the pseudocoelom appear to have a single polar flagellum (Fig. 9.1E). Although it is likely that these rod-shaped bacteria are *Pasteuria*, it has not yet be shown unequivocally as there are reports that helper bacteria may be involved in the growth of *P. penetrans* (Duponnois

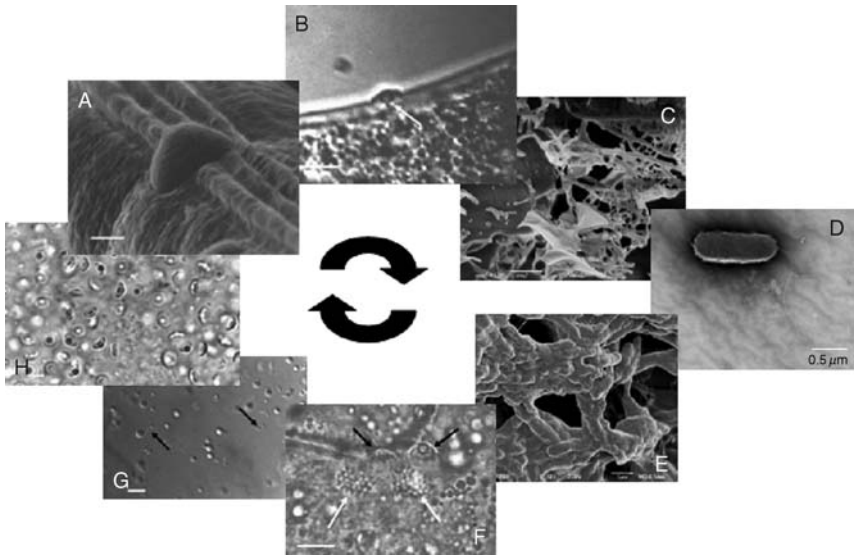

FIGURE 9.1 The life cycle of the parasite *Pasteuria penetrans* on its root-knot nematode host. (A) Scanning electron micrograph (SEM) of adhesion of endospore to the cuticle of an infective juvenile (bar = 1 μm); (B) light micrograph of infection peg (arrow) breeching the cuticle marking germination following formation of a feeding site by an infective juvenile (bar = 5 μm); (C) SEM showing rhizoids penetrating pseudocoelomic cavity (bar = 1 μm); transmission electron micrograph (TEM) of rod-shaped bacterium from within psuedocoelomic cavity with polar flagellum (bar = 0.5 μm); (E) SEM showing granular masses for bacterial rods within pseudocoelomic cavity (bar = 1 μm); (F) light micrograph of endospores (black arrows) on the infective juvenile cuticle surface and daughter microcolonies (white arrows) within pseudocoelomic cavity (bar = 5 μm); (G) light micrograph of fragmented microcolonies (doublets; black arrows) present in pseudocoelomic cavity (bar = 5 μm); (H) light micrograph of mature endospores from an adult infected female (bar = 4 μm).

et al., 1999; Gerber and White, 2001; Hewlett *et al.*, 2004). Root-knot nematode females infected with *P. penetrans* produce few, if any, progeny as their reproductive system quickly degenerates (Davies *et al.*, 2008). Sporogenesis begins when unidentified triggers, perhaps when certain key nutrients are limiting, with the production of microcolonies (Fig. 9.1F). These consist of clumps of dichotomously branching mycelial-like structures that subsequently fragment into quartets and doublets (Fig. 9.1G). This process continues until single, separate sporangia are produced, each containing a single endospore (Fig. 9.1H). An individual female that is infected can contain over 2×10^6 endospores and infected females after 6–8 weeks, often become larger than uninfected females (Davies *et al.*, 1988). This is similar to the situation in *P. ramosa* where infected *Daphnia* are larger than uninfected ones and gigantism has

been implicated (Ebert, 2005). The endospores are released back into the soil when infected nematodes and plant roots decay.

9.3. EXPLOITING GENOMICS TO UNDERSTAND THE *P. PENETRANS* ROOT-KNOT NEMATODE BIOLOGY

9.3.1. Sequencing plant-parasitic nematodes and *Pasteuria*

Over the last decade, the sequencing of eukaryotic and prokaryotic organisms has become routine. Comparing the genomes of different organisms can often lead to insights into their evolutionary history and help to answer questions regarding how organisms with similar developmental processes and genetics have very different life forms, and conversely, how very similar life forms can have very dissimilar developmental processes and genetics (Cañestro *et al.*, 2007; Frutos *et al.*, 2006). Computer software is being developed to make the comparisons between nematode species (Harris, 2003) and bacterial species (Field *et al.*, 2005) easy and accessible. Several plant-parasitic nematodes are currently being sequenced and these include *Meloidogyne incognita*, *M. hapla*, *Heterodera glycines* and *Globodera pallida*. In addition, there are a whole series of ESTs present within the public databases at GenBank and EMBL. The same is also true for *Pasteuria* and from a survey of the genome (Bird *et al.*, 2003) nearly 4,000 nucleotide sequences are available through GenBank and EMBL. Although at present there is no completed *Pasteuria* spp. genome available, at least one is very close to completion and new sequences are being deposited on a monthly basis. Even without having a completely sequenced genome, it is possible to start making comparisons between closely related species and gain an understanding into key biological processes. As *P. penetrans* has potential for being developed into a biological control agent, understanding of this particular host–parasite interaction is essential if it is ever going to be developed into a commercial control agent. There are two aspects that are currently prohibiting its commercial development: the inability to mass culture the bacterium *in vitro* and its restricted host range. Focusing on these two fundamental problems, it is therefore possible to gain insights from genomic comparisons of the host–parasite interactions that may help development of the bacterium as a biological control agent.

9.3.2. Genomic insights for *in vitro* mass production of *P. penetrans*

Up until very recently, the mass production of *P. penetrans* for the control of plant-parasitic nematodes has had to rely on *in vivo* culturing methods. The majority of these methods are adaptations of the method developed

by Stirling and Wachtel (1980). Briefly, females infected with *P. penetrans* spores are collected and an endospore suspension is made by homogenising infected females in water. Infective root-knot nematode juveniles are then exposed to these spores so that each juvenile is encumbered with 5–10 endospores. These encumbered second-stage juveniles are then placed around the roots of a tomato plant. After 6–8 weeks the nematode-infected roots containing infected nematodes are washed free of soil and air dried. The roots are then milled and can be used as inoculum for application to soil. Such milled tomato root powder can contain as many as 1.3×10^9 endospores per gram of root powder but the number of spores in each batch is highly variable (Pembroke and Gowen, personal communication). Although this is enough for application for small-scale growers, large-scale growers will require levels of mass production that would be better suited to an *in vitro* culturing method. Early attempts to grow *Pasteuria in vitro* (Bishop and Ellar, 1991; Williams *et al.*, 1989) produced very limited success. Bishop and Ellar produced two media, one of which would sustain vegetative growth and another led to the production of endospores, but because at no point did the bacteria grow exponentially they were never able to produce enough for commercial application. More recently, Pasteuria Bioscience LLC, Florida, has developed media in which it is possible to grow vegetative stages of *Pasteuria* (Hewlett *et al.*, 2004).

Both Bishop and Ellar and, more recently, Pasteuria Bioscience were unable to provide conditions in which vegetatively growing cells changed their growth form and entered sporogenesis. The initiation of sporulation in *Bacillus subtilis* has been extensively studied and is dependent on a phosphorelay pathway (Burbulys *et al.*, 1991). In this pathway, a phosphoryl group is transferred to the regulator Spo0F through a group of five kinases that are under environmental regulation. This phosphoryl group is then transferred to the phosphotransferase Spo0B, which in turn passes it onto the regulator/transcription factor Spo0A. Phosphorylation of Spo0A enhances the activation and repression of approximately 500 stationary phase and sporulation genes (Fawcett *et al.*, 2000). Like all known regulators, Spo0F requires a divalent metal ion to be present in the conserved aspartic acid pocket in order for phosphorylation to occur (Grimshaw *et al.*, 1998) and magnesium has been shown to be important (Zapf *et al.*, 1996). More recently, it has been suggested that metal cations other than Mg^{2+} may play a role in the structure and function of Spo0F and its involvement in the initiation of sporulation (Mukhopadhyay *et al.*, 2004). Investigations of the effects of the divalent cations Ca^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+} on the structure and function Spo0F of *B. subtilis* showed that they bound to the aspartic acid pocket and that while Mg^{2+} supports phosphotransfer from the kinase KinA to Spo0F the copper cation Cu^{2+} inhibited their phosphotransfer (Kojetin *et al.*, 2005).

Interrogation of the *Pasteuria* survey sequence (using BlastP) revealed a large number of genes (approximately 6%) that had a high degree of similarity to genes involved in sporulation (Bird *et al.*, 2003) and this included Spo0F. Alignment of Spo0F between *B. subtilis*, *B. anthracis*, *B. thuringiensis* and *P. penetrans* showed that key amino acids that form the aspartic acid pocket are conserved across these groups. From the results discussed above it was hypothesised that the presence of Cu^{2+}, at non-lethal concentrations in the sporulation media for *B. subtilis* and the related bacterium *P. penetrans*, might inhibit endospore formation while continuing to permit vegetative growth. Indeed, subsequent experiments revealed that the absence of Cu^{2+} in the media showed an increased number of sporulating cells (Kojetin *et al.*, 2005). This result suggests that the availability of Cu^{2+} could be used to induce vegetative cells to enter sporulation.

9.3.3. Endospore attachment to the nematode cuticle

9.3.3.1. Host specificity in *Pasteuria penetrans*

The initial infection of infective root-knot juveniles by *P. penetrans* endospores is determined by the ability of viable endospores to adhere to the cuticle of migrating nematodes in search of a host plant root. Therefore, the attachment is the primary and, arguably, the most fundamental step in the infection process. There is a large number of studies (Channer and Gowen, 1992; Davies *et al.*, 1988, 1990; Espanol *et al.*, 1997; Mendoza de Gives *et al.*, 1999b; Sharma and Davies, 1996; Stirling, 1985; Wishart *et al.*, 2004) that show that endospores from individual isolates of the bacterium do not adhere to or recognise all populations of nematodes and exhibit host attachment specificity. Indeed, it has been shown that cuticle heterogeneity as exhibited by endospore attachment is not linked in any simple way to the phylogeny of the nematode (Davies *et al.*, 2001) and, in addition, in standard attachment assays differences can also be found between different stages of the same nematode population (Davies and Williamson, 2006). Perhaps more intriguing is the observation that inter- and intra-specific functional variation as measured again by *Pasteuria* spore attachment assays showed an equal amount of variation between amphimictic and parthenogenetically reproducing species of root-knot nematodes (Davies *et al.*, 2008). It is important to understand the mechanism that determines host specificity in the bacterium in order to identify suitable populations of *Pasteuria* to control specific nematode pests and possibly identify bacterial strains with a wide host range.

9.3.3.2. The hair-like fibrils of *Pasteuria penetrans* endospores

The structure and function of bacterial endospores has recently been reviewed (Henriques and Moran, 2007). In many species, the endospore coat is the outermost layer, however, in other species the spore is

surrounded by an additional layer called the exosporium. *P. penetrans* possesses an exosporium, which in other species provides it with resistance to chemical and enzymic treatments and gives the spore its adhesive properties (Kozuka and Tochibuko, 1985; Takumi, *et al*., 1979). Fibrils are known to be important in the attachment of many bacteria to host surfaces and their decoration with sugars has been observed to confer host specificity (Benz and Schmidt, 2002; Power and Jennings, 2003; Takeuchi *et al*., 2003). The exposure of endospores to hydrochloric acid removes its central body to reveal a structure containing fibrils (Persidis *et al*., 1991) and scanning electron microscope (SEM) studies on intact endospores have revealed that the parasporal fibres are positioned in such a way around the central body of the endospore to produce a skirt-like structure in which the under-surface of the endospore is in intimate contact with the nematode cuticle (Fig. 9.2A).

On the processing of endospores for SEM, occasionally the central body of the spore falls away from the nematode revealing a circular membrane adhering to the nematode cuticle (Fig. 9.2B) that was an integral part of the parasporal fibril, skirt-like structure. Transmission electron micrographs (TEMs) of the parasporal fibres covered with fine fibres both on the upper and lower surface (Fig. 9.2B) and further observations have shown the fibres on the concave surface of the endospore are more densely distributed than on the upper surface (Fig. 9.2B). It is difficult not to conclude that these fibres are involved in attachment of the mature endospore to the nematode cuticle.

In species of other closely related bacteria, *B. cereus*, *B. thuringiensis* and *B. anthracis*, for example, the structure of the exosporium is species and strain specific (Plomp *et al*., 2005a,b) and on the outer surface of which is an external hair-like nap (DesRosier and Lara, 1981; Wehrli *et al*., 1980). This is similar in *P. penetrans*. In *B. anthracis* the hair-like nap appears to be formed by a single collagen-like protein BclA in which the length of the filaments is related to the number of G-X-Y repeats (Boydston *et al*., 2005; Sylvestre *et al*., 2002, 2003, 2005). Homologous genes to *bclA* have been identified in other *Bacillus* spp., and they reside in a rhamnose cluster operon that contains a number of glycosyltransferases forming an exosporium island (Charlon *et al*., 1999; Steichen *et al*., 2003; Todd *et al*., 2003). In a genomic survey of *P. penetrans* (Bird *et al*., 2003) collagen-like sequences were identified using BlastP against *B. anthracis*, *B. cereus* and *B. thuringiensis*, that they were phylogenetically closer in structure to bacterial collagens (Davies and Opperman, 2006). Three contiguous gene sequences were identified each containing 28, 36 and 87, collagen-like G-X-Y repeats from which it was possible to predict that the *P. penetrans* hair-like nap would be made up of filaments with lengths ranging from 56 to over 200 nm in length (Davies and Opperman, 2006). TEM studies of *P. penetrans* endospores have so far not provided evidence

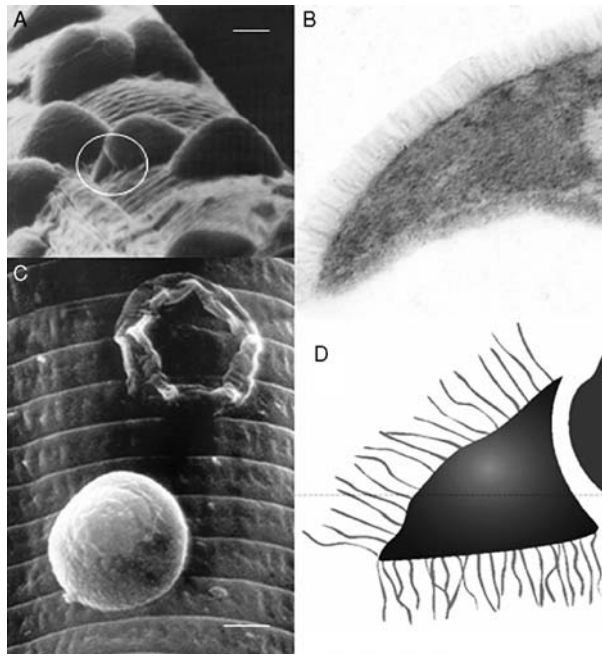

FIGURE 9.2 Endospore attachment (A) Scanning electron micrograph (SEM) of endospores adhering to the surface of an infective juvenile, ringed area reveals a point where the skirt-like structure that surrounds the central body of the endospore has broken away (bar = 1 μm); (B) transmission electron micrograph (TEM) of cross-section through the skirt-like structure that surrounds the central body of the endospore showing it to be covered in a hair-like nap the underside of which is the more dense than the upper surface; (C) SEM of an endospore and above a membrane-like structure that remains attached to the infective juvenile when the central body of the endospore has broken away during processing (bar = 1 μm); (D) cartoon (not to scale) of the skirt-like structure that surrounds the central body of the endospore and is covered with a fibrous nap.

of fibres with a length significantly greater than 100 nm but exosporial filaments ranging in length from 20 to over 100 nm have been identified (van de Meene *et al.*, unpublished data). Evidence that these fibres on the surface of the endospore are collagen-like come from the facts that endospores incubated in collagenase are reduced in their ability to attach to the nematode juvenile cuticle (Davies and Danks, 1993), and that endospores pre-treated with either fibronectin or, perhaps more significantly, the collagen-binding domain of fibronectin, are also inhibited in their ability to attach to the juvenile cuticle (Davies and Redden, 1997; Mohan *et al.*, 2001). However, confirmation of their structure will require further investigation.

9.3.2.2. The nematode cuticle and microbial adherence

The complex structure of the nematode cuticle reflects the multiple roles it has to perform, from protection of the nematode against the external environment, through to being important for nutrition and excretion, and to acting against the hydrostatic skeleton for locomotion (Wright, 1987). The cuticle of *C. elegans* is the most studied and best understood (Blaxter and Bird, 1997; Kramer, 1997; Politz and Philipp, 1992) and can therefore be used as a model. However, it should be remembered that the structure of the cuticle is highly variable among different groups and growth stages of nematodes (Malakhov, 1994). The nematode cuticle has consistently been divided into three easily definable layers, basal, medial and cortical layers, each of which is readily identifiable and easily observed by microscopy (Baldwin and Perry, 2004; Bird and Bird, 1991). The basal layer is typically striated and collagen is thought to be an important component; the medial layer is usually highly variable between species and frequently possesses struts, also made up of collagen, and upon which is the cortical layer. This outer layer appears to have a number of different specialised features, such as annulations and has been shown to contain cuticlins, lipids, surface-associated proteins and carbohydrates (Blaxter and Robertson, 1998; Cox *et al.*, 1981a,b; Himmelhoch and Zuckerman, 1978; Zuckerman *et al.*, 1979) and the binding of antibodies to this outer layer, the surface coat, affects nematode movement and behaviour (Sharon *et al.*, 2002). This surface coat differs fundamentally from the lower collagenous layers and the epicuticle, in that ethanol is sufficient to extract it (Page *et al.*, 1992). Because many standard electron microscopy techniques employ ethanol for dehydration, this means it is very difficult to observe.

Biochemical and immunological approaches have been used to characterise the surface coat of many animal-parasitic nematodes, and it is known that rapid changes in the surface coat can occur between pre-parasitic and post-parasitic forms (Proudfoot *et al.*, 1993). Hence, seen from this perspective, *C. elegans* is a poor model as it is not a parasite and until relatively recently very little was known about its surface coat. New information is now being obtained through genetic studies, which is much more advanced in *C. elegans* than in other nematodes. Recent genetic studies have revealed genes which, when mutated, have produced defects in the surface coat (Grenache *et al.*, 1996; Hemmer *et al.*, 1991; Link *et al.*, 1992; Politz *et al.*, 1990). These changes to the surface coat have also been associated with the ability of pathogens to adhere to the cuticle and set up microbial infections (Gravato-Nobre *et al.*, 2005; Hoflich *et al.*, 2004; Mendoza de Gives *et al.*, 1999a). In addition, wild-type *C. elegans* are susceptible to the formation of bacterial biofilms around the head of the nematode that prohibit feeding, and mutation experiments have identified a number of surface mutants in which the biofilm is absent

on the head (Darby *et al*., 2002, 2007). This suggests that surface coat properties are under direct genetic control and are important in the adherence of pathogenic and non-pathogenic bacteria.

Many of the genes identified by mutagenesis experiments that are associated with the bacterial adherence have a role in glycosylation pathways. Glycosylation occurs at the Golgi apparatus or endoplasmic reticulum where proteins can be decorated with sugars, a process that requires glycosyltransferases. Many of the mutants that have been identified with alterations in bacterial adherence encode genes involved with nucleotide sugar transporters and glycosyltransferases (Darby *et al*., 2007; Hoflich *et al*., 2004; Yook and Hodgkin, 2007). Mucin-like glycoproteins are important molecules that appear to be involved in host–parasite interactions and have been found in a variety of nematode species (Gems and Maizels, 1996; Loukas *et al*., 2000; Tetteh *et al*., 1999; Theodoropoulos *et al*., 2001). Mucins are a family of polypeptides associated with both the innate and adapted immune systems and can be secreted or membrane bound to form a protective barrier that covers epithelial surfaces (Strous and Dekker, 1992). Mucins possess a polypeptide backbone, parts of which are highly glycosylated with sugar side chains making up 85% of the molecule's weight. Glycosylation is predominantly *O*-linked through *N*-acetylgalactosamine (GalNAc) to serine and threonine residues within a VNTR region of the polypeptide core (Hicks *et al*., 2000; Theodoropoulos *et al*., 2001). Although there is no information on the role of mucins in plant-parasitic nematodes, it is interesting that the genes *muc-2*, *muc-3* and *muc-4* (which are members of the TES-120 family of proteins present in *Toxocara canis* (Tetteh *et al*., 1999) and are responsible for surface coat variation and have homologues in *C. elegans*) are also all present in various species of plant-parasitic nematodes (Table 9.1).

9.3.2.3. Microvilli and plant-parasitic nematode cuticles

The cuticle of plant-parasitic nematodes is broadly similar in structure to that discussed above; it can be divided into three layers, the outer most of which, the cortical layer, contains an epicuticle that is covered with the surface coat. The surface coat has a fuzzy appearance (Wright, 1987) and is composed mainly of proteins, carbohydrates and lipids (Blaxter and Robertson, 1998; Spiegel and McClure, 1995). This outermost layer is an important structure in that it provides a barrier between plant-parasitic nematodes and their environment. The second-stage juvenile is the infective stage of root-knot nematodes and thus the surface coat is exposed to two very different environments: a) the soil, as juveniles migrate in search of a plant root and b) the plant root, which they enter and through which they migrate to find a position to establish a feeding site. In the soil the infective juvenile will need to defend itself against microbial pathogens (Davies, 2005) and when it enters a host root it will need to remain

TABLE 9.1 TBlastN[1] of *Toxocara canis* surface coat glycoproteins (mucins) to top hit to plant-parasitic nematodes (E-value $<1e^{-10}$)

Toxocara mucin	Plant-parasitic nematode spp.	Score	E-value	% Similarity	Account number
T-120 (U39815)	*G. rostochiensis*	204	$2.6e^{-15}$	55	BM354635
	H. glycines	187	$1.6e^{-13}$	69	BI749309
	H. schachtii	185	$2.7e^{-13}$	65	CF100132
	M. hapla	170	$1.3e^{-11}$	81	BM901418
	G. pallida	169	$2.3e^{-11}$	52	BM416491
Muc-2 (AF167707)	*M. javanica*	208	$8.0e^{-16}$	53	CF350929
	M. chitwoodi	197	$1.5e^{-14}$	45	CD420128
	G. rostochiensis	189	$1.0e^{-13}$	59	BM354635
	M. hapla	187	$1.2e^{-13}$	49	CN194142
	H. glycines	160	$1.2e^{-10}$	67	BI749309
Muc-3 (AF167708)	*M. javanica*	273	$1.0e^{-22}$	49	CF350929
	M. chitwoodi	246	$9.5e^{-20}$	51	CD420128
	M. hapla	232	$3.1e^{-17}$	58	CN194142
	H. glycines	206	$1.6e^{-15}$	66	BI749309
	G. rostochiensis	192	$4.3e^{-14}$	56	BM355263
Muc-4 (AF167709)	*M. hapla*	236	$1.2e^{-18}$	60	CN194523
	H. glycines	227	$9.5e^{-18}$	67	BI749309
	M. chitwoodi	229	$6.0e^{-18}$	48	CD420128
	G. rostochiensis	226	$1.1e^{-17}$	64	BM355263
	M. javanica	221	$3.3e^{-17}$	54	CF350929
	H. schachtii	210	$6.1e^{-16}$	70	CF100132

Note:[1] complexity filter removed due to repetitive Ser and Thr repeats in core polypeptide. BLAST searches undertaken at: http://www.nematode.net/BLAST/.

undetected by the plant (Jones and Robertson, 1997; Kaplan and Davis, 1987). An important property of the surface coat is its lability, and the fact that the infective juvenile has to contend with two very different environments, the soil and the plant root, suggests that its ability to shed and regenerate its surface (Bird and Zuckerman, 1989; Gravato-Nobre *et al*., 1995, 1999, 2001; Lin and McClure, 1996) is important to its survival. Therefore, as in animal parasitic nematodes (Almond and Parkhouse, 1985; Blaxter *et al*., 1992), the surface coat plays an important role in actively evading infectious microbial agents and the immunity of the host plant.

Early studies using the outer layer of the cortex of some insect parasitic nematodes have been studied using TEM and have revealed finger-like projections or microvilli (Riding, 1970). Subsequent studies have suggested that the presence of two other types of cuticle, one where the secretory activity of the hypodermis had produced a covering or surface coat under which was a layer of microvilli, and another where the microvilli were fused and interwoven with a secreted surface coat (Subbotin *et al*., 1993, 1994). TEM studies of infective juveniles of the plant-parasitic nematodes *Globodera rostochiensis*, *Meloidogyne incognita* and *Heterodera glycines* have also revealed fibrillar and/or microvilli like projections that were thought to be secreted (Endo, 1993; Forrest *et al*., 1989; Fig. 9.3). The fixation of nematodes for electron microscopy is considered difficult and the use of alcohol in the preparation process can have very detrimental effects on surface structures. New techniques employing cryofixation and high-pressure freezing of *C. elegans* has produced images where microfibrial structures are observed on top of which is a surface coat that varies in thickness (Müller-Reichert *et al*., 2003). Endospores of

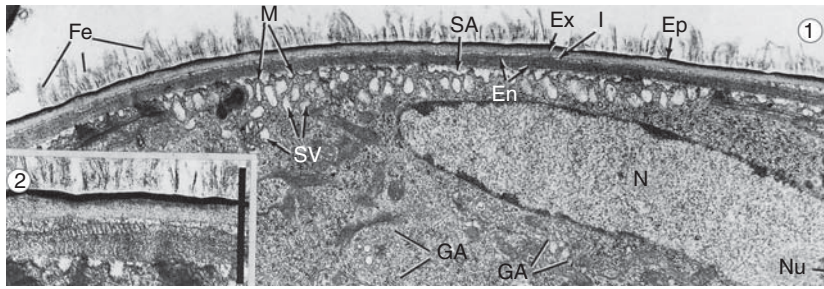

FIGURE 9.3 Longitudinal section of J2 at 2 days after inoculation. Figure shows fibrillar exudates (*fe*) on cuticle surface near stylet region. Section through hypodermal cord shows elongated nucleus (*N*) and extensive accumulation of secretory vesicles (*SV*) adjacent to apical membrane (*M*) of hypodermis (*H*). *En*, endocuticle; *Ep*, epicuticle; *Ex*, exocuticle; *GA*, Golgi apparatus; *I*, intermediate zone; *Nu*, nucleolus; *SA*, secretion accumulation zone. Bar = 1.0 μm (adapted from Endo, 1998; Figs. 162 and 163).

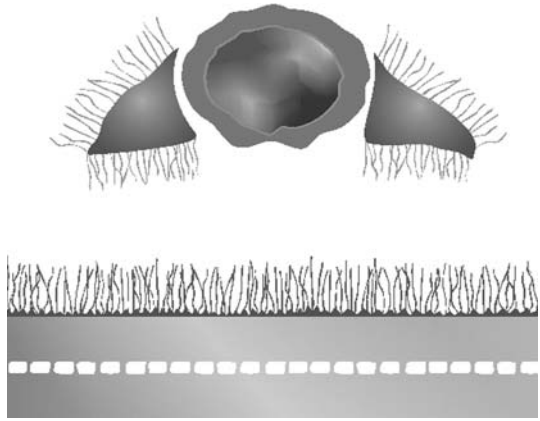

FIGURE 9.4 Cartoon (not to scale) showing endospore of *Pasteuria.* The skirt-like structure that surrounds the central body of the endospore is covered in a fibrous hair-like nap that interacts with fibrous structures present on the surface of the nematode cuticle. It is proposed that endospores attach to the nematode by a Velcro-like mechanism (see text Section 9.3.3).

Pasteuria are known to attach to the surface of infective juveniles of *H. glycines* (Atibalentja *et al*., 2004) and it is reasonable to suggest that the collagen fibrils present on the surface of the endospore, as discussed above, are either directly or indirectly interacting, with the microvilli present on the surface of the infective juvenile using what can be described as a Velcro-like attachment mechanism (Fig. 9.4). As *Pasteuria* endospores have been seen adhering to a large number of plant-parasitic nematodes (Chen and Dickson, 1998) this would suggest that the presence of microvilli on the surface of nematodes is likely to be a more common phenomenon than is currently appreciated. However, the fact that a population of endospores will not attach to all plant-parasitic nematodes (Davies *et al*., 2001) suggests that the surface coat has some system of generating variability that produces the observed host specificity.

9.3.4. Endospore specificity and the Velcro-like model for attachment

The Velcro-like mechanism of endospore attachment described above suggests that collagen-like fibres similar in structure to BclA in *B. anthracis* and *B. thuringiensis* are between 20 and 120 nm in length, and as migrating infective juveniles come into contact with endospores lying dormant in the soil it is likely that the *Pasteuria* collagen-like fibres will bind to the microvilli that form a part of the nematode's surface coat. Mucins are molecules that have a high degree of glycosylation, and are reported to

have an important role in host–nematode interactions (Hicks *et al*., 2000; Theodoropoulos *et al*., 2001). As reported above, mucins present in *T. canis* have homologues present in plant-parasitic nematodes and, arguably, it can be hypothesised that it is likely that similar mucin-like molecules may be important in the attachment of endospores to the nematode cuticle. Attachment of endospores to the infective juvenile cuticle is highly specific (Channer and Gowen, 1992; Davies *et al*., 1988, 1990; Espanol *et al*., 1997; Mendoza de Gives *et al*., 1999b; Sharma and Davies, 1996; Stirling, 1985; Wishart *et al*., 2004) and monoclonal and polyclonal antibodies raised to the surface of a population of *Pasteuria* endospores showed the surface characteristics of these spores to be diverse (i.e., different subpopulations of an initial population of *Pasteuria* would adhere to different populations of root-knot nematode, revealing a diversity of the surface coat of the cuticle to which the endospores were adhering) (Costa *et al*., 2006; Davies and Redden, 1997; Davies *et al*., 1994). This raises the question of the molecular nature of host specificity and the genetics that defines it.

9.3.3.1. Importance of carbohydrates in *Pasteuria*–nematode interactions

Mucins are polypeptides that are highly decorated with sugars (Hicks *et al*., 2000; Theodoropoulos *et al*., 2001), the addition of which can have both a physio-chemical and a biological function. The addition of carbohydrate domains to protein can modify solubility, electrical charge, mass and viscosity, and can control protein folding and three-dimensional stability, as well as protect it from enzymatic digestion (Lis and Sharon, 1993). Biologically, glycosylation can determine the lifetime of a protein and regulates its movement and position in the cell; it is also highly important in cellular interactions where it can be important in cellular recognition and determine a cell's antigenic characteristics (Lis and Sharon, 1993). Glycosylation is ubiquitous in eukaryotes and recently there have been increasing reports of glycosylation pathways in bacteria, particularly among mucosal-associated pathogens (Szymanski and Wren, 2005). To investigate the role that carbohydrates may play in endospore attachment, infective juvenile nematodes and endospores were pre-treated with a series of glycolytic enzymes and their effect on endospore attachment quantified (Davies and Danks, 1993). Pre-treatment of infective juveniles had a much greater effect on reducing endospore attachment than the pre-treatment of endospores, suggesting the importance of carbohydrates on the second-stage juvenile cuticle. Interestingly, in the same study, the pre-treatment of endospores with periodic acid, a treatment that breaks the hexose sugar ring and therefore disrupts the epitope, but does not break the saccharide chain (Maizels *et al*., 1991), had a greater effect in reducing attachment than that of pre-treatment of cuticle (Davies

and Danks, 1993). This result suggested that a carbohydrate–protein interaction was responsible for the specificity of the adhesion. However, recently atomic force microscopy has shown that equally strong adhesive forces between two glycan molecules can be obtained, as between proteins in antibody–antigen interactions (Bucior *et al.*, 2004). This report leaves open the possibility that carbohydrate–carbohydrate interactions may play a major role in regulating the specificity of endospore attachment to the nematode cuticle. Some sugars have been shown to inhibit endospore attachment; pre-treatment of the infective juvenile cuticle of *M. incognita* with *L*-fucose, *N*-acetylglucosamine and *D*-xylose all reduced endospore attachment by 50% or more, whereas other sugars, *D*-glucose, *N*-acetylneuraminic acid, *D*-galactose, *D*-mannose and *N*-acetylgalactosamine had no or very little effect (Davies and Danks, 1993).

9.3.3.2. Molecular mimicry and fucose

In an investigation of the role of the surface coat in nematode–plant interactions, it was shown that a monoclonal antibody raised specifically against the outer surface of infective juveniles of *M. incognita* recognised a fucosyl-bearing glycoprotein and that this glycoprotein was sloughed off and deposited along the migratory track of the nematode as it migrated within the plant root (Gravato-Nobre *et al.*, 1999). It has been suggested that plant-parasitic nematodes share antigens with their host plant (McClure *et al.*, 1973) and indeed the nematode surface coat appears to share a fucosyl-bearing epitope with the phloem elements of nematode-infected roots (Gravato-Nobre *et al.*, 1999). However, fucose is present in the side chains of xyloglucan, a component of the plant cell wall (Masuda *et al.*, 1989), but the anti-fucose antibody did not recognise the fucosyl-moieties in roots that were not infected by nematodes. This suggests that either the fucose is of nematode origin, or, if of plant origin, it only becomes available for labelling as a result of infection of the roots by nematodes. If the antigen is of nematode origin can be hypothesised that the secreted antigen is involved in antigenic mimicry as a form of molecular camouflage and/or it is a signalling molecule where it may be responsible for the induction of the host response (Gravato-Nobre *et al.*, 1999).

Following on from the published evidence reviewed above, it could be hypothesised that the carbohydrates involved in the decoration of collagen-like fibrils on the surface of the endospore, and the glycosylation of the core mucin peptides of the nematode surface coat are responsible for the host specificity observed between *Pasteuria* endospores and infective juvenile nematodes. In addition, these same decorated mucins of the nematode surface coat may also be important in the regulation of specificity between the nematode and its plant host. In an investigation of glycosylation in Gram-negative bacteria representing a wide evolutionary

distance (Power and Jennings, 2003), it was shown that there were a number of conserved features: 1) where the protein target was known the genes responsible for glycosylation are adjacent to the protein they are to glycosylate, 2) there is a common arrangement of the biosynthetic genes necessary for glycosylation that suggests a common evolutionary origin, 3) the occurrence of acyl carrier proteins occur in groups of genes where proteins need to be glycosylated and therefore suggests they have a role, and 4) a large number of polymorphisms and the presence of mechanisms for phase-variable expression of glycosylation genes within a strain suggests periodic immune or functional selection for variation in glycan structure. These common features suggest the genes involved with glycosylation are within islands that can generate variable polymorphisms that are the consequence of an on-going host–parasite arms race (Dawkins and Krebs, 1979). Therefore, these conserved features might be expected to be present in the genomes of *Pasteuria* and plant-parasitic nematodes and with the publication of the root-knot nematode sequence (Opperman *et al.*, 2008) and imminent publication of the *Pasteuria*-sequencing project this hypothesis will be open for rigorous testing.

9.3.3.3. Evidence of molecular diversity to produce polymorphism

The attachment of endospores to the cuticle of infective juveniles is a key interaction that will have co-evolved in the context of a host–parasite molecular arms race to produce polymorphism and molecular diversity. The evidence that this has occurred can be seen in the host specificity observed in endospore attachment studies and where this has been investigated further using immunological approaches.

Monoclonal antibodies (Mabs) raised to endospores from a single host female of *Pasteuria* endospores, strain PP1, produced five Mabs that showed that there was a diversity of surface types as different subpopulations of the endospore of strain PP1 were recognised by each of the five different Mabs (Davies *et al.*, 1994). Baiting the PP1 population of endospores with different species and races of root-knot nematode and using the Mabs to characterise the endospores that were adhering to each of the different populations of nematode, showed that different subpopulations of the endospores were adhering to the different nematode populations. This indicates that immunological heterogeneity in the surface of the endospore was related to heterogeneity present in the outer surface coat of the different nematode populations (Davies *et al.*, 1994). Similar differences were also observed in the recognition of the surface of endospores between isolates of *Pasteuria* from different geographical regions by the different Mabs. One particular Mab, PP1/117, appeared to recognise the concave surface of the endospore to a greater extent than the upper surface, revealing that the density of the antigen was greater on the concave surface; pre-treatment of these endospores with sugars or

glycolytic enzymes reduced the ability of the Mab to bind suggesting the Mab was recognising a carbohydrate epitope (Davies and Redden, 1997). Interestingly, the greatest reduction in recognition by pre-incubation of the spore in a carbohydrate by Mab PP1/117 was by fucose (Davies and Redden, 1997), which, as discussed above, may be involved in molecular camouflage of the infective juvenile to evade a plant host response. Other pre-treatments of the endospore that had a significant effect (>70%) on Mab PP1/117 recognition were proteinase K, fibronectin, wheat germ agglutinin and *n*-acetylglucosamine (Davies and Redden, 1997).

In Section 9.2.3 above, a Velcro-like molecular model is proposed that involves glycosylated collagen-like fibres on the surface of the endospore interacting with mucin-like peptides present on the outer surface coat of the infective juvenile. The evidence above points to the fact that glycosylation of the core collagen and mucin peptides that are present on the endospore and nematode cuticle, respectively, may be a source of polymorphism that determines the specificity of attachment. There may be other aspects of this molecular interaction that may also be important in maintaining the polymorphism. *P. penetrans* has been shown to have genes that encode for different lengths of the collagen-like protein (Davies and Opperman, 2006). The lengths of these collagen-like endosporal fibres in *Pasteuria* may well also be strain specific and play a role in attachment specificity. Thus, it is likely that collagen length and structure, core mucin length and structure, together with glycosylation, combine together to account for host range and specificity observed between endospores and nematode cuticle.

9.4. BUILDING COHESION BETWEEN MOLECULES AND POPULATIONS

9.4.1. Endospore heterogeneity and the density necessary for nematode suppression

P. penetrans has been identified as a key organism contributing to the suppression of plant-parasitic nematodes in a number of situations (Davies *et al*., 1990; Giblin-Davis *et al*., 1990; Mankau, 1975; Minton and Sayre, 1989; Oostendorp *et al*., 1991; Weibelzahl-Fulton *et al*., 1996). It has been argued that it has potential to be developed into a biological control agent and there are a number of worked examples where this has been achieved on a wide number of crops in different situations (Bhattacharya and Swarup, 1988; Chen *et al*., 1997; Chen *et al*., 1996; Chen and Dickson, 1998; Stirling, 1984; Trudgill *et al*., 2000). Nevertheless, there are many anecdotal reports where *P. penetrans* has been applied but has not successfully controlled the nematode population. Clearly, the ability to attach

to and subsequently infect the nematode target is fundamental. It has been estimated that for a soil to become suppressive, or for nematode control to take place, 10^4 endospores per gram of soil are required (Davies *et al*., 1990; Stirling, 1991). However, these estimates do not take account of the fact, as reviewed above (Section 9.2.2.), that there is a large amount of surface heterogeneity present within each endospore population, which could be potentially important in determining endospore adhesion. Therefore, for any particular nematode population there will only be a sub-population of the 10^4 endospores per gram of soil that will indeed attach to and infect any particular nematode population.

From the perspective of biological control, the amount of inoculum required is a key constraint, and it could be argued that 10^4 endospores per gram of soil is an overestimate of the number of spores necessary to suppress nematode populations. This is because what will be important in understanding this interaction at the population level is not the total numbers of nematodes or endospores, but the relative proportions of compatible and non-compatible nematode–*Pasteuria* interactions and the underlying genetics responsible for the interaction. Both immunological and DNA-based methods have been developed to characterise and quantify *P. penetrans* (Costa *et al*., 2006; Davies *et al*., 1994; Davies and Redden, 1997; Duan *et al*., 2003; Preston *et al*., 2003; Schmidt *et al*., 2004; Sturhan *et al*., 2005) and root-knot nematodes (Davies and Carter, 1995; Davies and Lander, 1992; Davies *et al*., 1996; Powers, 2004; Tigano *et al*., 2005) in soil, but relating this intra-specific variation to host–parasite compatibility and understanding the genetics involved has so far remained elusive. However, the co-evolutionary interaction between *Daphnia* and *P. ramosa* has been an active area of study and perhaps this will provide insight into the plant-parasitic nematode/*Pasteuria* interaction.

9.4.2. Learning from *Daphnia–P. ramosa* interactions

9.4.2.1. *Daphnia* and its parasitism by *P. ramosa*

Daphnia spp. are small transparent crustaceans found in most freshwater ponds and lakes that feed on plankton that can reproduce sexually and asexually but under normal conditions females reproduce by apomictic parthenogenesis (under unfavourable conditions a female will produce haploid eggs that need fertilisation by asexually produced sons). *Daphnia* can live for 2–3 months with the first eggs being formed after 7–15 days at 20 °C and can easily be maintained in the laboratory (Ebert, 2005). *P. ramosa* is a parasite of *Daphnia* that produces gigantism and sterilises the host shortly after infection, in a similar manner to *P. penetrans*, but whereas the route of infection is well understood in nematodes, it is not well understood in *Daphnia*. In *Daphnia*, it has been demonstrated that a strong host genotype–parasite genotype interaction exists (Carius *et al*.,

2001; Ebert, 1994; Refardt and Ebert, 2007), and as with *P. penetrans* there are no *Pasteuria* isolates that are able to infect all host genotypes (Carius *et al*., 2001) and even from within the same infected host isolates of *P. ramosa* could be obtained that exhibited different amounts of virulence (Little *et al*., 2008).

Epidemiological models usually assume that the mass action model applies and that the number of susceptible individuals that become infected is a result of the density of the host and the concentration of the parasite (Regoes *et al*., 2003). Recently significant deviations from this basic model have been observed and attributed to biotic and abiotic factors such as seasonality, temperature, spatial structure and non-genetic host heterogeneity with respect to immunity and susceptibility (Ben-Ami *et al*., 2008; Vale *et al*., 2008). However, taken as a whole, none of these non-genetic factors were as strong as the genetic effects, and only genetic effects have been shown to explain variation in resistance under natural conditions (Ebert, 2008; Little and Ebert, 2000). Interestingly, in a comparison of *P. penetrans* endospore attachment to single juvenile descent lines of sexual and asexual reproducing root-knot nematodes, even within clonal lines there were significant differences in endospore attachment, suggesting some special mechanism was operating that produced functional differences in the cuticle surface that affected endospore attachment (Davies *et al*., 2008). Therefore, similar to the *Daphnia* system, where the variation in the interaction is not all accounted for by genetics, perhaps this is also true for the root-knot nematodes, where some, as yet, unspecified non-genetic mechanism is contributing to host heterogeneity.

9.4.2.2. 'Arms races' and the 'Red Queen' hypothesis

It is difficult to study from a co-evolutionary perspective the reciprocal interactions that occur between hosts and parasites in a natural system because of the many generations needed. Recently the *Daphnia–Pasteuria* system was shown to offer a rare opportunity because lake sediments contain a unique archive due to the fact that both *Daphnia* and *Pasteuria* produce dormant propagule banks. Samples from different age strata in the sediments can be revived and then accessed using infectivity and virulence assays between different populations from the age-stratified sediments to assess 'Red Queen' co-evolutionary dynamics (Decaestecker *et al*., 2007). The 'Red Queen' hypothesis (Van Valen, 1973) is based on the concept that within an antagonistic 'arms race' (e.g., between a predator and its prey, or a host and its parasite), where an increase in fitness of the host (e.g., genes for resistance against a parasite) will lead to a reciprocal increase in fitness in the parasite (e.g., new virulence against its host). This naturally leads to what has been regarded as an 'arms race' between a host and its parasite. Although the results from the *Daphnia–P. ramosa* system did not show any change in

parasite infectivity over time, there was a continued decrease in fecundity of the host and an increase in *Pasteuria* endospore production; these results were interpreted as an increase in the virulence of *P. ramosa* (Decaestecker *et al.*, 2007). These results clearly show that infection needs to be separated from virulence, and, interestingly, different life-cycle strategies can be observed between different species of *Pasteuria* that infect different host nematodes.

The spores of *P. penetrans* that infect root-knot nematodes do not germinate until the nematode has entered the plant and set up a feeding site. An individual nematode can produce over 2 x 10^6 endospores (Davies *et al.*, 1988), which greatly contrasts with the *Pasteuria* population that infected *Heterodera avenae* where spores attached to infective juveniles germinate immediately, kill their host rapidly and stop infected individuals from migrating; these infected individuals produce fewer than 1,000 endospores per infected individual (Davies *et al.*, 1990). These observations, in light of the results reported by Decaestecker *et al.* (2007), suggest that there may be a co-evolutionary development from a necrotrophic lifestyle to a more biotrophic one, where it could be argued that two contrasting strategies, which probably form two extremes of a continuum, have evolved: 1) where the host is killed rapidly and few endospores are produced, with another, 2) where the nematode's life span is maintained or even extended together with the production of a large number of endospores. Indeed, there is now growing evidence in the *Daphnia–P. ramosa* system that the 'trade-off' hypothesis is at work, and that the evolution of virulence has led to a situation in which the production of endospores is balanced with the exploitation of the host in such a way that lifetime transmission success, production of endospores, is maximised (Jensen *et al.*, 2006).

9.5. A MOLECULAR APPROACH TO INFECTION AND VIRULENCE FROM AN EVOLUTIONARY PERSPECTIVE

As can be seen above, there is a growing literature on understanding the interaction between *Pasteuria* spp. and their respective hosts, both at the molecular level and at the population level and this knowledge needs to be brought together. Molecular biologists might argue that phenomena at the population level can be explained by the facts at the lower level, a 'bottom up', or reductionist, approach; while the ecologist might argue that phenomena at the population level cannot be explained from knowledge of the parts, and must be studied directly, a 'top down', or holistic approach (Maynard-Smith, 1986). Therefore, the challenge for the 'pure' scientist is to bring the knowledge of these two approaches together and integrate them, while the challenge for the applied scientist is to utilise

this knowledge and apply it to solve problems. Many polymorphisms are maintained by the interactions of hosts with their parasites (Haldane, 1949). This idea was developed by plant pathologists and formalised within what is known as the gene-for-gene hypothesis (Flor, 1956, 1971). The concept of gene-for-gene co-evolution has been brought into question as it has been suggested that it is an artefact of studies undertaken using agricultural examples (Thompson and Burdon, 1992). This is a consequence of studies using spatially, or in the case of potato cyst nematode (*Globodera* spp.) in the United Kingdom, geographically isolated populations, and not natural populations. Nevertheless, the gene-for-gene model, along with a number of other conceptual models such as 'matching-genotypes', 'quantitative-trait', 'multiplicative matching-alleles' and 'additive matching-alleles' models have been found to be useful in developing our understanding (Nuismer and Otto, 2004; Otto and Nuismer, 2004). Mathematics has proved useful in understanding host–parasite interactions in relation to the Red Queen hypothesis and the evolution of sex and different levels of ploidy. Within this context, it is interesting that *H. avenae*, parasitised by a *Pasteuria* sp. that germinates and reproduces in the infective juvenile, is diploid and amphimictic, while *M. incognita* where *Pasteuria* normally germinates in the developing females causing gigantism, is polyploid and mitotically parthenogenetic. Until more comparative life-cycle studies have been undertaken investigating other nematode–*Pasteuria* associations and their genetics, this just remains an interesting observation.

Biochemical recognition systems are important in determining which matching host and parasite genotypes result in controlling resistance and susceptibility (Frank, 1994). The polymorphic diversity revealed by endospore attachment, where this manuscript proposes a form of Velcro-like mechanism of attachment, probably involving nematode mucins on the cuticle and *Pasteuria* collagen-like fibres that are glycosylated on the endospore, is likely to be under direct genetic control. However, whether or not this is directional and the result of a Red Queen arms race remains to be ascertained because other methods of generating variation, such as gene rearrangement and reading frame shifts (de Vries *et al*., 2002; Weiser, 2000), error prone DNA polymerase (Rattray and Strathern, 2003) and post-translational modification (Hicks *et al*., 2000; Theodoropoulos *et al*., 2001) can also produce phenotypic variation. The importance of the genetics that underpins these interactions will only be clarified when the biochemical and molecular mechanism of the attachment process is fully understood. Endospore attachment, however, only represents the first stage in the infection process. The germination of endospores is also important and differs between the *Pasteuria* parasitising different hosts. This suggests different signalling processes are probably operational at different stages in the infection process and it is likely that natural

selection will have produced another level of specificity. Finally, once infection has successfully taken place, the virulence of the bacterium is also important and it has been observed that *P. ramosa* strains isolated from the same host have been shown to have very different levels of virulence against an isolate of *Daphnia* (Jensen *et al*., 2006). Attachment, germination and virulence are all likely to be important in the co-evolved adaptations of *Pasteuria* to its nematode host. It will, therefore, be the subtleties of these interactions that will determine the outcomes of different *Pasteuria* spp. relationships with their nematode hosts.

9.6. SUMMARY, CONCLUSIONS AND IMPLICATIONS FOR BIOLOGICAL CONTROL

It is only relatively recently with the development of industrialised agriculture that plant-parasitic nematodes have been recognised as an important constraint on crop production. For the majority of their evolutionary history, plant-parasitic nematodes have been part of a multi-trophic interaction between their plant host, and their natural enemies. The biotic constraints to their population growth will be through several mechanisms: 1) top-down control, exerted by their natural enemies; 2) horizontal control, exerted by inter- and intra-specific competition among the nematodes, and 3) bottom-up control exerted by their host plant (Van der Putten *et al*., 2006).

This manuscript concentrates on top-down control by the obligate hyperparasitic bacterial group of natural enemies that are from the endospore-forming genus *Pasteuria*. The life cycle of the bacterium is described in which migrating infective juvenile nematodes become encumbered with *Pasteuria* endospores that infect the nematode and prohibit it from reproducing. There are two problems in developing *Pasteuria* as a biological control agent: 1) its obligate nature and the inability to mass culture it and 2) its host specificity.

Genomic approaches have been useful in gaining insights into both *in vitro* culture and host specificity. Knowledge of the sporulation pathway of closely related *Bacillus* spp. is well developed and highly conserved across different groups of endospore-forming bacteria. This has provided useful insights into the cation concentration required in growth media that are conducive for sporogenesis. Genomics has also been used to understand endospore specificity.

The first stage of the infection process is the attachment of endospores to the cuticle of the infective juvenile and this is highly host specific. Reviewing the literature and gaining insights from sequencing data suggests a Velcro-like mechanism of spore attachment. A molecular model is proposed in which glycosylated collagen-like fibres on the surface of the

endospore interact with fibrous mucins present in the surface coat of the infective juvenile. It is this process where the collagen and mucin fibres act as the primary architectural structures for attachment and where glycosylation is likely to be involved in determining host specificity.

The *Daphnia–Pasteuria* model is then drawn upon to show that even from within the same *Daphnia* host isolate, different levels of virulence can be observed in different *Pasteuria* strains and that there is an observable relationship between the life spans of infected hosts and the number of endospores produced that suggests that there is a co-evolutionary development. A similar difference in life-cycle strategies can also be seen in plant-parasitic nematodes infected with *Pasteuria*.

The development of knowledge about the *Pasteuria*–nematode interaction has provided insights into two problems that prohibit *Pasteuria* from being developed into a commercial control agent, culturing and host specificity. As more is learnt through genomic approaches, and our understanding of the co-evolutionary processes that have shaped different life-cycle strategies between different strains of *Pasteuria* grow, this knowledge will help in the development of novel and new strategies of plant-parasitic nematode control.

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CHAPTER 10

Host–Parasite Relations and Implications for Control

Alan Fenwick

Contents

Abstract

This paper considers the various measures available to control several of the neglected tropical diseases (NTDs). To develop the optimum methods for controlling the parasites that cause these NTDs, knowledge of the life cycles of both the parasites and their vectors are essential. Each NTD requires its own strategy for control based on detailed knowledge of the life cycle, and vector control, chemotherapy, better water supplies and better hygiene are all components that may be appropriate. For some diseases, improved drugs are urgently required, for some the tools are

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available for elimination, while uniquely guinea worm could be eradicated without any chemotherapeutic drug being used. Several NTDs lend themselves to mass drug administration (MDA) in which human populations are annually offered safe, effective and usually donated drugs with a view to morbidity control and/or elimination. The drugs could and should be used to improve the quality of millions of lives, prevent suffering, stigma, disfigurement and early death. The role of pharmaceutical companies who have donated their drugs for the treatment of millions of disadvantaged people in the developing world is acknowledged. One result of such drug pressure however is that evolutionary change may result, and it is incumbent on scientists during monitoring and evaluation of control programmes to ensure that such changes are recognised. One other unfortunate development is that a paucity of newly trained vector-borne disease experts may constrain future control efforts.

10.1. INTRODUCTION

This paper considers the optimum methods for the control of neglected tropical diseases (NTDs). NTDs is a recently conceived collective name for a group of diseases, some vector-borne and some non-vector-borne, which as the name suggests have been neglected - which means they have not been considered a priority in resource poor countries, and therefore have not been controlled. They are mostly parasitic diseases that are particularly prevalent in tropical developing countries, and affect the poorest people usually living in rural areas. The major 14 NTDs are listed in Box 10.1, and given the appropriate public health tools most of these diseases should be easy to prevent or control and some should be close to elimination in specific confined areas. However, the control of parasitic diseases depends on a thorough knowledge and understanding of the life cycle and the biology of the parasite and of its hosts. Using that knowledge, strategies for control can be developed based on available tools. For several diseases, chemotherapy can be used to kill the parasite in the human host, but employing other strategies, including better hygiene and sanitation or vector control, should be complimentary to chemotherapy if appropriate (Hotez *et al.*, 2007). In this paper public health control measures used to control each disease and how these measures are developed from a knowledge of the life cycles are described.

The major parasitic diseases include one (malaria) which dominates the minds of health professionals in Africa particularly where it is still estimated that malaria is responsible for 1 million deaths a year (Greenwood *et al.*, 2005). Apart from malaria, the 14 major neglected tropical diseases (NTDs) include parasitic helminths and protozoa but

BOX 10.1 Major neglected tropical diseases

- Protozoan infections
  - Leishmaniasis (VL + CL + MCL)
  - African trypanosomiasis (sleeping sickness)
  - Chaga's disease
- Helminth infections
  - Soil-transmitted Helminth infections:
    - Ascariasis-trichuriasis-hookworm
  - Lymphatic filariasis (elephantiasis)
  - Onchocerciasis (river blindness)
  - Schistosomiasis
  - Dracunculiasis (guinea worm)
  - Cysticercosis
- Bacterial infections
  - Leprosy
  - Trachoma
  - Buruli ulcer

also bacterial infections (Box 10.1) (Hotez *et al*., 2006). The main causes of death in the developing world are the acute diseases caused by HIV-AIDS, malaria and TB, and these have been the focus of significant efforts to improve global health for over 20 years. Their control programmes have received substantial funding through the Global Fund, (Poore, 2004) the United States Presidents Malaria Initiative (Loewenberg, 2007) and PEPFAR (Hanefeld, 2008), although the ambitious targets set for these programmes have yet to be reached. Meanwhile, until very recently the NTDs were being truly neglected (Fenwick *et al*., 2005). However when the acute and chronic consequences caused by these NTDs is aggregated, the collective burden of disease, particularly in children of school age, is as great as malaria, and TB, and approaches that of HIV/AIDs. These childhood infections lead in later life to stigma (Weiss, 2008), to long-term disabilities, and an early death (Fenwick, 2006). What is regrettable, is that in 2008, a large percentage of the disease burden caused by most NTDs is now unnecessary because simple, inexpensive, safe and effective tools for their control or even elimination exist (Fenwick, 2006).

However, there is not one simple solution for all the NTDs, because each organism requires a specific strategy of attack, because each has a different vector or transmission mechanism, different epidemiology, or different criteria for treatment strategy. For each disease a 'trigger

prevalence' is recommended by the World Health Organization, which means that if in a survey the prevalence is found to be above that 'trigger prevalence', then control measures (usually MDA) are justified. Indeed for each different NTD the control programmes will have different target outcomes, either morbidity control, transmission control, elimination or even eradication. These triggers and targets however, are relatively newly defined, because until recently these NTDs have either not been subject to national control programmes, or have been controlled in vertically funded programmes (Hotez *et al.*, 2006).

10.2. THE CURRENT STRATEGIES FOR NTD CONTROL

Since 2006, the donors supporting NTD control (The Bill and Melinda Gates Foundation and some bi-lateral donors), the World Health Organization, the pharmaceutical industry, and the managers of these vertical programme have worked towards integration of their efforts leading to a consolidated approach to NTD control. Thus a rapid impact package of drugs has been advocated for control of seven of the NTDs (Hotez *et al.*, 2006). One disease, lymphatic filariasis, LF, has been targeted for elimination (Ottesen, 2006), while for the others (onchocerciasis, schistosomiasis, trachoma and three soil transmitted helminths, STH) containment of disease is the prime short-term objective. Another helminth (Guinea Worm) lends itself to eradication using simple hygiene and clean water interventions (Al-Awadi *et al.*, 2007). Trachoma elimination is also a realistic possibility provided MDA is part of a combined (SAFE) strategy, where the SAFE elements are Surgery, Face washing and Environmental improvements. The 'A' is the antibiotic Zithromax that is donated by Pfizer to treat existing infections (Kumaresan, 2005).

For LF, the WHO suggests that the best control strategy for elimination is mass drug administration (MDA) annually of a combination of albendazole co-administered with either ivermectin or DEC (which cannot be given where onchocerciasis is co-endemic for safety reasons). Programmes against onchocerciasis, schistosomiasis and STH are currently aiming to control the disease morbidity caused by infections since elimination is not considered a viable target at this time. The drugs used are, respectively, ivermectin, praziquantel and albendazole or mebendazole (Hotez *et al.*, 2006).

The relationship between the parasites and its hosts and/or vectors, the possibilities for vector control, transmission control, and the efficacy of existing treatments must be considered when developing a strategy for control.

10.3. CONTROL OF EACH NTD

10.3.1. Guinea worm

Guinea Worm (*Dracunculiasis)* is different from most of the helminth infections in that there is no treatment for the adult worm in the human, and so effective transmission control is essential. However, transmission control has become an effective tool because of our knowledge of the life cycle, which is dependent on the human host ingesting the copepod intermediate host. The fact that humans are the only definitive host makes eradication using early diagnosis, case control, clean water and improved hygiene a real possibility (Ruiz-Tiben and Hopkins, 2006). The Guinea Worm eradication strategy therefore depends on interrupting transmission in both directions. First, new human infections can be prevented immediately by stopping any ingestion of copepods, simply by filtering all drinking water whether from well or pond water to remove all copepods. Filtration may vary from the simplest of cloth filters through which all drinking water is passed before drinking, to provision of a piped, filtered and treated mains supply. Meanwhile copepods are killed *in situ* by ABATE larvicide application monthly in ponds, pools and unprotected wells during the transmission season. Second, the prevention of eggs from adult worms from humans reaching copepod-infested water will thereby prevent intermediate hosts from becoming infected, which will prevent transmission. This can be achieved only when the gravid Guinea Worm emerging from the human host is quickly recognised, and the case correctly controlled, so that all patients with gravid worms are prevented from washing themselves and their emerging worms in water. Because there is no chemotherapeutic cure for Guinea Worm in humans, early detection is essential so that when the gravid female starts to emerge, it can be teased out, and withdrawn by wrapping the emerging end of the worm around a stick and slowly pulling it out over time. With these simple measures, eradication is thus possible because humans and copepods are the only and obligatory hosts and transmission can only occur if people swallow the infected intermediate host fleas, when drinking from unfiltered water bodies. Special praise must be directed to WHO, CDC and the Carter Center for the progress made towards eradication. In a more recent recognition of how close Guinea Worm is to eradication, the British Government announced in September 2008, an allocation of £10 million to assist partners to finally eradicate Guinea Worm.

10.3.2. Lymphatic filariasis

After leprosy, lymphatic filariasis arguably causes the most horrific disfigurements and suffering of any disease on the planet, as the adult worms in the lymph nodes block the lymph system, and secondary

infections cause legs and breasts to swell. In men hydrocoeles can grow to obscene proportions. Millions upon millions are at risk of infection in Africa, the Indian sub-continent and the Far East, and over 120 million were estimated to be infected in 2000 (Ottesen, 2006). The adult worms produce microfilariae that circulate in the blood waiting to be ingested when the host is bitten by a female mosquito, and that mosquito will then infect the next person bitten. The mosquitoes, which are vectors of LF, are of course also the target for control in their own right because of the nuisance and disturbance due to their biting, but also because their *Anopheles* species transmit malaria. Against night biting mosquitoes long lasting insecticide impregnated bed nets will protect sleepers against LF, malaria and the nuisance bites during their time in bed. However, different mosquitoes have different biting habits, and different species take their blood meal during non-sleeping times, and so other measures to prevent bites are needed.

It has been shown that transmission of LF can be prevented by population-based chemotherapy, which prevents microfilariae circulation in peripheral blood, and hence vector infections (Ottesen, 2006). The current control strategy against LF therefore is based on mass drug administration (MDA) where whole populations are targeted with albendazole given together with ivermectin (where onchocerciasis is co-endemic) and DEC in areas where onchocerciasis is absent. The global efforts to eliminate LF are arguably the single most effective coming together of partnerships. Drug companies, GlaxoSmithKline (GSK) and Merck each donate their products (albendazole and ivermectin, respectively) and delivery is funded by international donors and local governments, managed by NGOs and implemented by community drug distributors. The scale is enormous, with 750 million doses of albendazole donated during the first 10 years of the GSK donation programme (http://www.gsk.com/infocus/lf.htm) by January 2008. Merck donate almost 70 million doses of ivermectin annually for onchocerciasis control, and have donated over 150 million doses for the LF programme (http://www.merck.com/cr/enabling_access/developing_world/mectizan/). Over 500 million people were treated with albendazole and either DEC or ivermectin against LF as part of national elimination programmes in 2007. However, over 25 countries in Africa still need to implement a national programme, but expansion is on-going, and with the move towards integration of vertical programmes it is hoped that funds for further expansion of coverage in the poorest countries in Africa will continue. In a separate development, in two studies one in Zanzibar (Mohammed *et al.*, 2008) and another in Nigeria (Richards *et al.*, 2006), triple therapy with praziquantel, ivermectin and albendazole has satisfactorily passed large scale safety trials, and this should lead to further integration of NTD control programmes.

To alleviate existing suffering however, the case management of LF still needs more extensive health education and resources to offer limb washing for long-term infected individuals and surgery against hydrocoeles (Addiss and Brady, 2007). Eventually bed nets against malaria will hopefully be more widespread, and this should reduce transmission in parallel with the MDA thus eliminating this horrific disease (Molyneux, 2008).

10.3.3. Onchocerciasis

The causative agent of river blindness is a worm that lives in nodules in the human host and is difficult to kill *in situ*. The microfilariae that these worms produce cause symptoms of itching and eventually blindness as the worms cross the eye. Approximately 85.5 million people in Africa, Latin America and the Arabian Peninsula are at risk of contracting onchocerciasis. Globally, approximately 18 million people are infected, 1 million are visually impaired, and more than 350,000 have been blinded by infection. Approximately 95% of all infected people live in Africa (Ndyomugyenyi, 1998). The vector is the blackfly (*Simulium* sp.), which breeds in fast flowing water. With our understanding of the dynamics of transmission and the biology of the vector, this disease was one of the first to be attacked by human intervention against the vector on a large scale. Until DDT use was banned in 1973, *Onchocerciasis* vectors (*Simulium spp*) were controlled effectively during the Onchocerciais Control Programme (OCP) using DDT applied aerially into the major rivers in West and East Africa. Their regular application opened millions of hectares of land to settlement in areas of West and Central Africa where previously there had been blindness in a high percentage of riverside communities. However because that control involved the widespread aerial spraying of DDT into rivers in West Africa there were extreme environmental consequences, and this control tool, though effective, is no longer considered. Nevertheless, some 19 countries in West Africa are still free of river blindness due to this vector control, subsequent vigilance and focal ivermectin treatments (Hopkins, 2007).

Vector control preceded the widespread use of ivermectin, a drug which when dispensed annually to populations exposed to infection, reduced the microfilarial levels, relieved the terrible itching and prevented blindness. However, those who would benefit from ivermectin could never afford to purchase it and so in 1986 a major open ended donation programme was initiated by a pharmaceutical company, Merck. The control of onchocerciasis in areas where the parasite is still transmitted, is currently by the annual delivery of ivermectin, donated by Merck through the Mectizan Donation Programme (Thylefors, 2008) through community delivered mass drug administration. Thus while the control and even elimination of the insect vector is possible, it is no longer

attempted because of environmental considerations. In the previously OCP countries, ivermectin is delivered where there is still evidence of transmission or infection, and in other endemic countries in Africa the coverage with annual ivermectin is being increased to provide more widespread relief from infection with onchocerciasis (Hopkins, 2007).

10.3.4. Schistosomiasis

Schistosomiasis is caused by trematode worms (genus *Schistosoma)* which infect almost 200 million people globally and cause severe damage to the urinary system (*S. haematobium)* and liver (*S. mansoni* and *S. japonicum).* The parasites can only be transmitted from human to human via an appropriate species of aquatic snail in Africa and amphibian snail in Asia. However, the parasite has a huge reproductive potential and so with the increase in water projects in Africa in particular, and the explosion of the population together with an increase in life expectancy, schistosomiasis, previously considered a relatively benign disease, has become a disease of major public health importance. This is accentuated where selected favourable environmental conditions prevail, such as man made lakes and irrigation schemes, which have created vast new snail habitats and attracted human populations to settle close to attractive fresh water. The lack of piped water supplies and poor hygiene have created conditions for massive transmission, and people with heavy worm infestations have begun to suffer serious consequences earlier in life as liver and bladder-related symptoms caused by excessive egg deposition have taken their toll. Senegal suffered a massive schistosomiasis epidemic after the Diama Dam was constructed (Sow *et al*., 2002), and the most recent example to be watched is the Three Gorges Dam across the Yangtze River which will substantially change the ecology of the Dongting Lake in southern China and expand snail habitats (Li *et al*., 2007).

From 1920 through to 1980, snail control was considered to be the main tool available for transmission control, and the snail was considered the weak link in the life cycle (Fenwick, 1987). In Africa, snail control can in theory be achieved by a number of means—removing water bodies completely by drainage, replacing irrigation canals with pipes; changing the water bodies to make them less habitable to host snails, by cleaning out the weeds upon which snails feed, increasing flow or concrete lining; introducing biological control measures, such as predators (fish or ducks) or competitor snails. Chemical molluscicides such as copper sulphate, trifenmorpf, and niclosamide were used in certain control programmes (Fenwick, 1987) over a period of many years but they adversely affect the environment, and the best molluscicide, niclosamide, kills fish, and is also expensive. Numerous plant molluscicides have been tested (Kloos and McCullough, 1982), but none are in regular use to scale. Experimental

molluscicide applications soon showed that aquatic snails have a high reproductive potential and quickly re-populate after chemical control has been used (Fenwick, 1987). In China, manual collection of amphibious snails was tried with some reported success during the regime of Chairman Mao. Today, sadly, our knowledge of host biology leads us to reject snail control as a primary control tool because it appears to be environmentally unacceptable, ineffective and usually expensive. Instead we rely heavily on chemotherapy and MDA to control schistosomiasis, because of the emergence in 1980 of praziquantel—a 'wonder drug' against schistosomes—which although initially expensive—is in 2008, relatively inexpensive, effective and free of serious side effects (Fenwick, 2006). There are no recorded proven cases of resistance although anecdotal evidence of some tolerance has been reported (Doenhoff *et al*., 2002; Fallon *et al*., 1996; Ismail et al., 2002). The results of several years of MDA have shown dramatic improvements in health, and reductions in prevalence (Kabatereine *et al*., 2007; Koukounari *et al*., 2007). However, it is generally accepted that eventually only socio-economic improvement and provision of clean water supplies and sanitation will lead to elimination of schistosomiasis as a public health problem. Transmission can only be broken with better hygiene to prevent infected urine and faeces reaching snail infested water, and clean water supplies reducing the amount of water contact which leads to cercariae (the infective free swimming larvae which have developed in the snails) penetrating human skin (Fenwick, 2006).

10.3.5. Soil-transmitted helminths

The soil-transmitted helminths include ascariasis (*Ascaris luinbricoides)*, trichuris (*Trichuris trichuria*), and hookworm (*Ancylostoma duodenale* and *Necator americanus*). They do not utilise intermediate hosts, but thrive in different environmental conditions, and knowledge of their host parasite behaviour helps to devise control strategies. The adult worms each inhabit the human intestine, but the eggs that they lay follow different routes to infecting back into their human host. Both ascaris and trichuris eggs develop in the environment and depend on unhygienic behaviour to find themselves ingested by a human. Hookworm on the other hand are more 'pro-active' as larvae develop in the excreted eggs, then hatch out in the environment and then when ready, actively seek a human host by climbing grass and lying in wait for a passer by. They are capable of attaching to and penetrating human skin, migrating to the intestine where the adult worm attaches to the gut wall and completes the life cycle. Chemotherapy will remove most if not all the worms from the intestine (albendazole is said to be more efficient that mebendazole) (Albonico *et al*., 1999). The easiest regime might be annual mass chemotherapy in

school-aged children, but in known heavy wormy areas two treatments a year if feasible would be more efficacious. However improved hygiene and particularly efficient disposal of faecal material will control the transmission, and children wearing socks and shoes will certainly reduce if not prevent infection from hookworms.

10.3.6. Other NTDS

There are other vector transmitted parasitic diseases that are not controllable by mass chemotherapy, and these include leishmaniasis, trypanosomiasis and Chaga's disease transmitted by sandflies, tsetse flies and reduviid bugs, respectively.

10.3.6.1. Leishmaniasis

The leishmania organism causes two distinct diseases. Cutaneous leishmaniasis an unpleasant and disfiguring skin disease that causes suppurating sores that are self-limiting and self-heal after about 6 months. There is an animal reservoir in dogs. The visceral form of the disease however is a killer, and causes terrible suffering and certain death if untreated as the organism attacks the internal organs.

The leishmania parasite is carried by sandflies that are difficult to control, and outbreaks of leishmaniasis are likely in any areas of sandfly infestation, especially as these flies are too small to be deterred by a mosquito net. Regular out breaks of cutaneous leishmaniasis occur in towns and cities sited in desert areas (Khartoum in Sudan being one example) (el-Safi and Peters, 1991). Visceral leishmaniasis has a widespread distribution in North-Eastern China, India, Middle-East, Southern Europe (Mediterranean basin), Northern Africa, Central-East Africa and, in foci in Brazil and Honduras. Leishmaniasis control depends on sandfly control using insecticides, and in the case of visceral leishmaniasis, diagnosis and treatment with traditionally pentavalent antimony compounds and more recently amphotericin b.

10.3.6.2. Sleeping sickness (*Trypanosoma* spp.)

The disease in man is fatal if untreated. The two trypanosomes that cause sleeping sickness in man, and a related parasite that infects animals are all transmitted by the Tsetse fly (*Glossina spp*). The distribution of Tsetse flies is fortunately limited to a belt across central Africa, but in Tsetse areas, programmes of diagnosis and treatment are essential. This is one disease where vector control could be and should be implemented because a knowledge of the vector biology allows relatively easy control both by chemical and biological methods. Release of sterile males has had some success in the past, and Tsetse traps strategically placed in known breeding places can be effective. A recent innovative method of sleeping

sickness control has been to protect cattle using impregnated bandages that kill Tsetse flies that try to feed on these hosts (Fevre *et al*., 2005). These techniques have proved to be very successful in Uganda where both *T. rhodesiense* and *T. gambiense* exist and their habitat seems to be converging (Picozzi *et al*., 2005). The diagnosis and treatment of sleeping sickness are difficult because cases tend to be in remote areas where medical care is minimal. Mobile clinics are, therefore, the best way for prevention, diagnosis and treatment, though they depend on dedicated staff, and the use of unsatisfactorily dangerous drugs. A new drug against sleeping sickness is needed.

10.3.6.3. Chaga's disease (*Trypanosoma cruzi*)

Chaga's disease, which is confined to South America, should be doomed to extinction. However it is estimated that as many as 8 to 11 million people in Mexico, Central America, and South America may have the disease, although most of them do not know they are infected. If untreated, infection is lifelong and can be life threatening.

The parasite is transmitted by reduviid bugs (bed bugs) which live in cracks in walls in lower quality (mud) houses, and as their name suggests bite at night. Chaga's disease is easy to control because the vector is susceptible to indoor residual spraying with insecticides, and more effectively, these bugs should be eliminated by the simplest and basic improvement in socio economic conditions which should reduce or eliminate the bed bugs as housing quality improves. Sadly many people still live in relatively poor housing in South America and until their socio-economic status improves, the disease will not be eliminated.

10.3.6.4. Trachoma

While not strictly a parasitic infection, the causative agent of blinding trachoma is transmitted by insects, and the strategy for control is under the MDA umbrella of the rapid impact package of drugs for NTD control. Blinding trachoma is caused by the long-term consequences of infection with *Chlamydia* carried to children's eyes by flies, but the infection develops only because of poor hygiene, lack of water and therefore insufficient face washing. The untreated infection causes eyelid damage and subsequent corneal damage as distorted eyelashes scratch the cornea. A multi-targeted approach is necessary because while medicaments can prevent and treat early active infections, only surgery can prevent sufferers with existing eyelid damage from losing their sight. Vector control will prevent infections, because improved hygiene will reduce flies and face washing will prevent flies from infecting children.

10.4. CONSEQUENCES OF INTENSIVE THERAPY ON PARASITE EVOLUTION

Monitoring and evaluation of all the control programmes against all the NTDs is essential to prove to the donors and to the governments that the programme is effective, and delivers the promised drugs to the populations in need. It is also important to demonstrate that there are no adverse effects of the MDA. Thus it is important that all programmes have an element of monitoring that measures the effect of the MDA on prevalence and intensity of infection, and on clinical and nutritional status. In addition, while celebrating the rapid success achieved to date by such programmes, in terms of reduced infection prevalence, intensity and associated human morbidity, it is acknowledged that evolutionary change in response to drug selection pressure may be predicted under certain circumstances, across the different various NTDs, particularly in terms of the development of potential drug resistance, evolutionary changes in parasite virulence, transmission and host use, and/or competitive interactions with co-infecting pathogens. Theoretical and empirical data gained to date serve to highlight the importance of careful monitoring and evaluation of parasites and their hosts whenever and wherever chemotherapy is applied and where parasite transmission remains.

Thus the implementation of mass chemotherapy, will result in intensive and prolonged new selective pressures being placed on the parasite, which may have implications for the long-term success of such campaigns. The potential evolution of drug resistance is a particular pertinent issue for schistosomiasis because praziquantel is the only available drug. Against *S. japonicum* there seems to be no evidence of resistance to praziquantel after the clinical treatment of patients in China despite its wide use (Shi and Li, 2004), However, against *S. mansoni* concerns do seem justified that development of potential tolerance and/or resistance of schistosomes to praziquantel is possible (Danso-Appiah and De Vlas, 2002; Doenhoff and Pica-Mattoccia, 2006).

Since there has been so much treatment in several countries in Africa, and some isolates have been found, why have resistant worms not become of more significant importance. One possibility is the 'cost of resistance', whereby resistant genotypes are less fit than their susceptible counterparts in the absence of drug pressure, thereby preventing their establishment and spread—a hypothesis supported by theoretical studies (Orr, 2006; Xua *et al.*, 2005). Lamberton *et al*. (2005) suggests this may be the case because reduced cercarial production and increased virulence to the intermediate host appears among resistant *S. mansoni* lines relative to their susceptible counterparts.

It is thus recommended that monitoring for the possible development of drug resistance is imperative, and observations for other changes in the parasite due to evolution as a result of drug pressure be incorporated in any research component linked to every control programme.

10.5. CONCLUSION

A thorough knowledge of the host parasite interactions and behaviour and epidemiology of parasitic infections are essential in order to develop a strategy for their control. The different examples cited demonstrate the differences between the parasitic diseases, the different approaches to their control, and the different achievable targets of the separate control programmes. Despite these different strategies it seems that if NTD control is to succeed, integration of the individual programmes will be essential. However a word of warning, trained entomologists and malacologists are needed to implement vector and intermediate hosts control, and the number of entomologists and malacologists retiring is not being matched by the number being trained. There is a danger that within a generation, or even a decade there will not be the experts who understand vector biology. The dependence on mass drug administration to control these parasitic diseases is understandable, but potentially dangerous because resistance may develop to the limited number of drugs available leaving the world with no alternative control tools to keep the populations at risk of parasitic diseases from further epidemics of infection.

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CHAPTER 11

Onchocerca–Simulium Interactions and the Population and Evolutionary Biology of *Onchocerca volvulus*

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Abstract

Parasite–vector interactions shape the population dynamics of vector-borne infections and contribute to observed epidemiological patterns. Also, parasites and their vectors may co-evolve, giving rise to locally adapted combinations or complexes with the potential to stabilise the infection. Here, we focus on *Onchocerca–Simulium* interactions with particular reference to the transmission dynamics of human onchocerciasis. A wide range of simuliid species may act as vectors of *Onchocerca volvulus*, each exerting their own influence over the local epidemiology and the feasibility of controlling/eliminating the infection. Firstly, current understanding of the processes involved in parasite acquisition by, and development within, different *Simulium* species in West Africa and Latin America will be reviewed. A description of how *Onchocerca* and *Simulium* exert reciprocal effects on each other's survival at various stages of the parasite's life cycle within the blackfly, and may have adapted to minimise deleterious effects on fitness and maximise transmission will be given. Second, we describe the interactions in terms of resultant (positive and negative) density-dependent processes that regulate parasite abundance, and discuss their incorporation into mathematical models that provide useful qualitative insight regarding transmission breakpoints. Finally, we examine the interactions' influence upon the evolution of anthelmintic resistance, and conclude that local adaptation of *Onchocerca–Simulium* complexes will influence the feasibility of eliminating the parasite reservoir in different foci.

11.1. INTRODUCTION

Understanding the interface between parasites and their hosts provides insight into the biology of the infection process and how this process contributes to shape interactions at the population level. Also, this interface provides the backdrop for selection as at various stages in the interaction there will be pressures on parasites to enhance their transmissibility, which may result in them manipulating or exploiting the interaction and in hosts responding in various ways that help fend-off infection, and/or minimise damage or deleterious effects on fitness. Host–parasite interactions, therefore, have the potential to contribute to the shaping of short- and long-term epidemiological patterns, parasite–host co-evolution, and evolutionary change under selection pressures exerted by control interventions. These interactions will take place at various scales, from the molecular and cellular levels to the organismal and population levels. The detection of organismal- and population-level interactions (as emergent properties) can inform research into the molecular and cellular underlying mechanisms.

In this review we focus on the manifestations of *Onchocerca–Simulium* interactions as density-dependent effects, acting upon the parasite infrapopulation within the flies and upon the flies themselves. We examine evidence to suggest that *Onchocerca* may exploit such interactions to enhance its transmission and discuss the consequences on onchocerciasis transmission of local adaptation in *Onchocerca–Simulium* complexes. Finally, we examine the consequences of these processes for the population biology, transmission dynamics and control of *Onchocerca volvulus*.

11.1.1. *Onchocerca–Simulium* complexes

The interactions between the spirurian nematode parasites of the genus *Onchocerca* (Filarioidea: Onchocercidae) and their haematophagous intermediate hosts have been most extensively investigated for *Onchocerca volvulus* Leuckart in its *Simulium* Latreille (Diptera: Simuliidae) vectors. It is in the context of human onchocerciasis that the term *Onchocerca–Simulium* complexes was first used by Duke *et al.* (1966) to refer to well-adapted parasite–vector combinations that result in the development and transmission of the local *O. volvulus* population (e.g., the savannah form of the parasite developing successfully within savannah species of *S. damnosum* Theobald *sensu lato* (s.l.) but less so within forest species, and vice versa). Through a series of cross-experimental infection studies, in which flies were fed on microfilarial carriers of the same (sympatric) and distant (allopatric) localities, investigation of such complexes encompassed *O. volvulus–Simulium* comparisons not only within West Africa

but also between West Africa and Guatemala (De Leon and Duke, 1966; Duke *et al.*, 1967), West Africa and northern Venezuela (Duke, 1970), Guatemala and northern Venezuela (Takaoka *et al.*, 1986a,b), and more recently between the northern and Amazonian foci within Venezuela (Basáñez *et al.*, 2000). The results suggest the operation of strong local adaptation between the parasite and its vectors within well-established endemic areas, as opposed to greater incompatibility in heterologous combinations. The relevance of these findings is two-fold; first, because they have important implications for our understanding of the potential for onchocerciasis to spread outside its current endemic areas (Basáñez *et al.*, 2000; Maia-Herzog *et al.*, 1999; Schiller *et al.*, 1984), and second because they raise the question as to what would have been the potential for invasibility of New World areas, with local anthropophagic simuliid fauna and unexposed human populations, by *O. volvulus* when first brought to the Americas from Africa during the slave trade. (For the concepts of species invasiveness and community invasibility we follow Richardson and Pyek, 2006.)

11.1.1.1. Vector complexes

The notion of *Onchocerca–Simulium* complexes shall not be confused with the concept of vector species complexes, of which *S. damnosum* s.l. is an example (Vajime and Dunbar, 1975). A species complex (or a complex of 'sibling' or 'cryptic' species) is a group of closely related species, which, although may satisfy the criterion of being reproductively isolated from each other, are not readily or reliably distinguishable on a morphological basis, necessitating the use of cytological, genetic and/or ecological attributes to distinguish between them (White, 1978). Chromosomal speciation is widespread in Simuliidae (Rothfels, 1989). There are 55 valid and distinct cytoforms known from the *S. damnosum* complex, making it the largest sibling species complex (Post *et al.*, 2007). Nine sibling species serve as vectors for *O. volvulus* in West Africa, albeit with different capacities (Boakye, 1993). These include a savannah-dwelling group (*S. damnosum* sensu stricto and *S. sirbanum*; Vajime & Dunbar), a forest-dwelling group (*S. squamosum* Enderlein and *S. yahense* Vajime & Dunbar) and a transition-zone (forest-savannah mosaic)-dwelling group (*S. sanctipauli* Vajime & Dunbar, *S. leonense* Boakye, Post & Mosha, and *S. soubrense* Vajime & Dunbar) (Boakye *et al.*, 1998). Cytogenetic (Boakye, 1993) and DNA sequence analyses (Tang *et al.*, 1995) have supported the division of the sibling species into these three groups, and the former has indicated that hybridisation between the siblings and introgression occur (Boakye and Meredith, 1993; Boakye and Mosha, 1988; Boakye *et al.*, 2000). For a discussion of vector complexes in Latin America, see Shelley (1991). Fig. 11.1 presents a map of Africa (with Latin America inset) showing

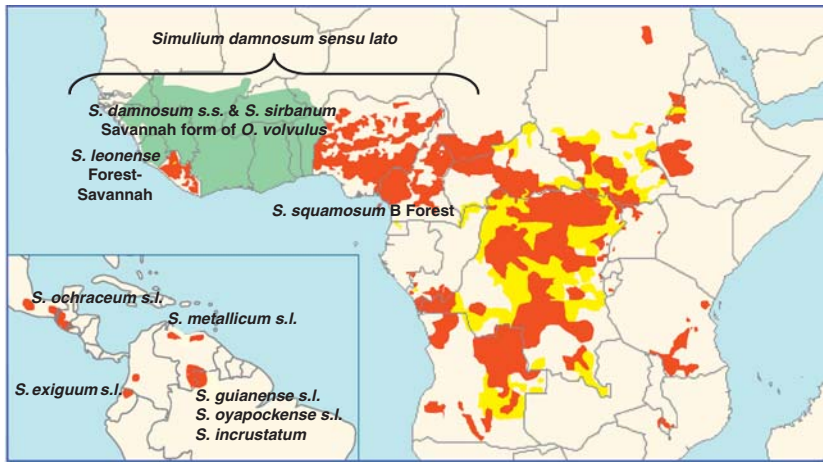


FIGURE 11.1 Distribution of human onchocerciasis endemic areas and vector species discussed in the text. The medium grey-shaded area represents the Onchocerciasis Control Programme in West Africa (OCP, 1975–2002); the dark grey areas depict regions presently undergoing mass ivermectin administration (annually in Africa, African Programme for Onchocerciasis Control (APOC, 1995–on-going) and biannually in Latin America, Onchocerciasis Elimination Program for the Americas (OEPA, 1993–on-going)); light grey areas require further rapid epidemiological mapping for onchocerciasis (REMO) surveys. Adapted from Basáñez *et al.* (2006).

the distribution of onchocerciasis, its control programmes and indicating the main vector species that will be discussed in this review.

11.1.2. *Onchocerca* in the Simuliid host

There are many opportunities for interaction between *Onchocerca* and its *Simulium* vector as the parasite undergoes considerable growth and development within the fly's thoracic muscles. Microfilariae must first be ingested with the blood meal, and a substantial portion of the blackfly's mouthparts is introduced into the skin of the definitive host by the cutting and piercing action of the mandibles and maxillae. Fig. 11.2 summarises the main stages in the process of infection at which the parasite encounters an interface with the vector which presents a challenge and an opportunity for interaction.

11.1.2.1. Ingestion of microfilariae

Contact between *Simulium* and *O. volvulus* begins with the ingestion of a blood meal containing microfilariae (the stage infective to the vector). When feeding, female simuliids locate the blood by cutting the skin of

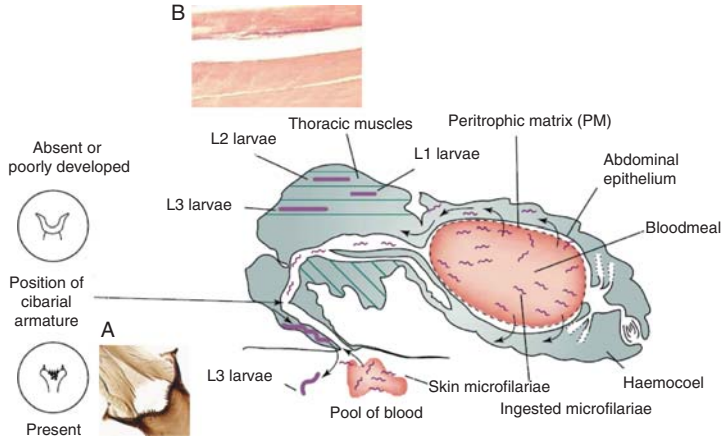


FIGURE 11.2 *Onchocerca* in the *Simulium* host. Microfilariae are recruited to the site of the wound caused by the piercing and cutting blackfly's mouthparts, and ingested with the blood meal (salivary secretions by the fly act on the vertebrate's haemostatic system and may attract microfilariae). Well-developed cibarial armatures (A) may cause lacerations to microfilariae, which otherwise must reach the haemocoel escaping imprisonment by the peritrophic matrix (PM). In the thoracic muscles (B), development of established microfilariae takes place to the infective stage in competent vectors. (A) Cibarium of *S. incrustatum* from the Brazilian part of the Amazonian focus; photograph taken by Luis Hernández, Natural History Museum, London. (B) Microfilariae of *O. volvulus* in the thorax of *S. metallicum* s.l. from north Venezuela; photograph taken from the archive of Jaime Ramírez-Pérez by Edmundo Guerrero, Tropical Zoology Institute, Universidad Central de Venezuela. Adapted from Basáñez and Ricárdez-Esquinca (2001).

the vertebrate host with rapid scissor-like movements of their mandibles, their maxillary laciniae penetrating downwards and creating a haemorrhagic pool upon which they feed (Ayesta *et al.*, 1985; Sutcliffe and McIver, 1984). While the hard mouthparts lacerate the skin, the membranous cuticles prevent, mechanically, blood loss from the wound and air entry into the food canal during feeding. The labral flaps within the food canal act as one-way valves, keeping blood from leaking out during the pumping down-stroke but allowing pooled blood to enter during the upstroke (Sutcliffe, 1985).

Simultaneously during this process, blackfly's saliva containing anti-haemostatic, pro-inflammatory, erythema-inducing, and immunomodulatory molecules (such as apyrase, anti-coagulants and anti-thrombin salivary protein) is injected into the host's skin at the site of the bite. These substances not only inhibit platelet aggregation, reduce coagulation

and induce vasodilation increasing blood supply to the feeding wound (facilitating easier and longer feeding), but the saliva's ability to modulate components of the host's immune system also provides an opportunity for enhanced parasite transmission during blood feeding (Andrade *et al.*, 2005; Cupp and Cupp, 1997; Hurd, 2003). Skin-dwelling *O. volvulus* microfilariae migrate towards the small pool of blood before being ingested by the blackfly and experimental evidence suggests that microfilarial orientation, movement and concentration into the feeding site may be mediated by a protein in the insect's saliva different from the erythema-inducing protein described earlier for New World simuliids (Stallings *et al.*, 2002). Although the hypothesis of whether *O. volvulus* could exploit the nature and activity of blackfly's salivary secretions to enhance its transmission must still be rigorously tested, in the following section the feeding experiment results that suggest this indeed might be an adaptive strategy in the *Onchocerca–Simulium* interaction will be summarised.

Cupp *et al.* (1995) reported that although apyrase activity inhibits platelet aggregation and is ubiquitous in blackfly's saliva, activity per gland varied according to species and had a positive association with degree of anthropophagy and *O. volvulus* vector status, being higher in *S. ochraceum* Walker than in *S. metallicum* Bellardi and *S. exiguum* Roubaud, with the lowest levels exhibited by the non-vectors *S. bivittatum* Malloch and *S. gonzalezi* Vargas & Díaz-Nájera. By contrast, anti-thrombin presence and activity also varied among blackfly species but exhibited a positive correlation with zoophagy (Cupp and Cupp, 1997). These authors also demonstrated that various simuliid species in the American continent differ in the vasodilator activity of their salivary secretions, with *S. ochraceum* s.l. exhibiting higher levels of such activity than *S. metallicum* s.l. Prior to this, working in Guatemala, Omar and Garms (1975) had recorded higher microfilarial intakes by *S. ochraceum* s.l. than by *S. metallicum* s.l., and De Leon and Duke (1966) had shown that microfilarial intake by *S. ochraceum* s.l. was also higher than that by *S. callidum* Dyar & Shannon. Shelley (1988), working in Ecuador, suggested that *S. quadrivittatum* Loew ingested more microfilariae than *S. exiguum* s.l., and Grillet *et al.* (2008) found, in the Amazonian focus of Venezuela, that *S. oyapockense* Floch & Abonnenc s.l. was able to ingest more microfilariae than *S. incrustatum* Lutz given the same microfilaridermia. These results suggest an inherent difference in the ability of various *Simulium* species to acquire microfilariae.

11.1.2.2. Survival of the microfilariae

The *O. volvulus–Simulium* interaction continues as blood is pumped into the stomach. In those species with a well-developed cibarial (sometimes referred as buccopharyngeal) armature, microfilariae may encounter rows of chitinous projections that protrude into the lumen of the foregut

as they are ingested together with the blood meal through the action of the cibarial and pharyngeal pumps located in the blackfly's head (Sutcliffe and McIver, 1984). Depending on the morphology of the armature, the projections may be more or less numerous, arranged in single or multiple rows, and blunter (papillae, rods) or sharper (cones, spines) in shape—there possibly is intra- as well as inter-specific variation in the armature's shape and size. In the context of onchocerciasis transmission in Africa and Latin America, two distinct groups of species are recognised according to the presence or absence of a well-developed armature (Basáñez and Ricárdez-Esquinca, 2001; Reid, 1978, 1994; Shelley, 1988). The former comprises species with cibarial 'teeth', including *S. ochraceum* s.l. and *S. haematopotum* Malloch in Meso American foci; *S. oyapockense* s.l., *S. incrustatum*, and S. *limbatum* Knab in the Amazonian focus, and *S. quadrivittatum* in Ecuador. The latter comprises species with poorly developed armatures, including the *S. damnosum* complex and the *S. neavei* Roubaud group in Africa, and *S. metallicum* s.l., *S. exiguum* s.l. and *S. guianense* Wise s.l. in Latin America.

Since cibarial armatures are more developed in the haematophagous females than in the non-blood-sucking males of certain families of biting flies, including Simuliidae, it has been proposed that the armature has mainly evolved in response to the blood-feeding habit, partly acting to prevent blood back-flow, and partly involved in the breaking up of erythrocytes to release the haemoglobin and other proteins prior to digestion of the blood in the abdominal mid-gut (Reid, 1994). However, secondarily and in terms of resistance to infection and simuliid survival, the cibarial armature serves as a first line of defence against ingested microfilariae (and parasite-induced fly mortality) by inflicting lacerations on the invading microfilarial stage.

Omar and Garms (1975) were the first to describe the consequences of the cibarial armature (of *S. ochraceum* s.l. in Guatemala) upon microfilariae of *O. volvulus* in contrast to the effect of an unarmed cibarium (of *S. metallicum* s.l.) and concluded that the armature damaged a substantial proportion of microfilariae en route to the stomach, where they disintegrated. Only 3% of the microfilariae migrated towards the thorax in *S. ochraceum* s.l. in comparison with 75% in *S. metallicum* s.l. (despite the former having ingested a significantly higher number of microfilariae than the latter). Omar and Garms' findings thus explained earlier observations by Bain *et al.* (1974) on the existence of 'two populations of microfilariae', one with the ability to migrate out of the stomach while the other is rapidly destroyed in an inverse proportion to the number of microfilariae ingested. Basáñez *et al.* (1995) suggested that Bain's observations could be explained by arguing that the fraction of microfilariae damaged by the cibarial armature could be density dependent, with a higher probability that ingested parasites are lacerated by the cibarial

teeth at lower intakes, and a smaller probability of damage at higher intakes (when some microfilariae may be protected by those that become entangled in the cuticular projections). Recent data from other 'armed' species (e.g., *S. oyapockense* s.l. and *S. incrustatum* in the Amazonian onchocerciasis focus) lend support to this hypothesis and indicate that the average damage caused is species specific (Grillet *et al.*, 2008). Interspecific variations in the shape of the cibarial armature and how these differences may be reflected in the degree of damage produced upon ingested filarial parasites have been observed in mosquitoes (McGreevy *et al.*, 1978). Table 11.1 presents a functional classification of simuliid vectors according to the presence/absence of a well-developed cibarial armature. All those (natural) armed vectors of human onchocerciasis are only in the Americas.

The fact that the cibarial armature, when well developed, can substantially reduce the number of microfilariae that are available for further migration to the thoracic muscles of the vector raises the question as to whether the parasite can overcome this constraint by exploiting the vector–parasite interaction to enhance other aspects of transmission. In the previous section we described the anti-haemostatic, anti-coagulant and vasoactive properties of blackfly's saliva on the host's blood (Cupp and Cupp, 1997). It is also possible that the parasite may utilise these properties of the vector–parasite interface to increase microfilarial intake by those simuliid species that destroy ingested parasites with their cibarial armatures. This could partly explain the results of the pair-wise comparisons listed above, in which the first species of the pair has been reported to exhibit higher microfilarial intakes than the second, namely, S. *ochraceum* s.l. (armed) versus *S. metallicum* s.l. (unarmed) (Omar and Garms, 1977); *S. quadrivittatum* (armed) versus *S. exiguum* s.l. (unarmed) (Shelley, 1988); *S. oyapockense* s.l. (more damaging armature) versus *S. incrustatum* (less damaging) (Grillet *et al.*, 2008).

11.1.2.3. Passage of microfilariae out of the blood meal

Lewis (1953) clearly described that the microfilariae of *O. volvulus* do not bypass an abdominal phase by reaching the thoracic muscles through direct migration from the thoracic mid-gut en route to the stomach. Rather, once in the abdominal mid-gut, microfilariae must leave the blood meal, reach the ecto-peritrophic space, traverse the abdominal epithelium, reach the haemocoel and migrate to the thorax, where they penetrate the muscle fibres and eventually grow and moult twice to become infective, L3 larvae. This abdominal phase therefore represents the next opportunity for *Onchocerca–Simulium* interaction. In response to the blood being ingested, a thick extracellular matrix (peritrophic matrix (PM), first described by Lewis (1950) for *S. damnosum*) is secreted (delaminated) by the abdominal mid-gut epithelium, completely surrounding

TABLE 11.1 Simuliid hosts of *Onchocerca* classified according to the presence or absence of a well-developed cibarial armature, their vector status and parasite density dependence

Locality	Type of cibarial armature[a]		Vector status[c]	Relationship between input microfilariae and output larvae[d]	References
	Well developed	Poorly or not developed			
West African savannah		*Simulium damnosum* s.s./ *S. sirbanum*	Natural	Limitation (negative density dependence)	Basáñez *et al.*, 1995; Philippon and Bain, 1972
West African forest (incl. forest-savannah mosaic)		*S. leonense*	Natural	Proportionality (density independence)	Soumbey-Alley *et al.*, 2004
		S. squamosum B[b]	Natural	Proportionality	Demanou *et al.*, 2003
East Africa		*S. neavei*	Natural	N/A	
Mexico and Guatemala		*S. callidum*	Natural	N/A	Dalmat, 1955
	S. haematopotum		Experimental	N/A	Takaoka *et al.*, 1986a
		S. metallicum A–K, X[b]	Natural	N/A	Shelley, 1991
	S. ochraceum A–C[b]		Natural	Initial facilitation (positive density dependence)	Basáñez *et al.*, 1995; Collins *et al.*, 1977
	S. veracruzanum		Experimental	N/A	Shelley, 1991
Colombia		*S. exiguum* s.l.	Natural	N/A	Tidwell *et al.*, 1980; Wetten *et al.*, 2007

Ecuador		*S. exiguum* Cayapa[b]	Natural	Limitation	Collins *et al.*, 1995; Wetten *et al.*, 2007
		S. exiguum Aguarico[b] Bucay[b] Quevedo[b]	Experimental	N/A	Shelley *et al.*, 1989, 1990; Wetten *et al.*, 2007
	S. quadrivittatum		Natural	N/A	Vieira *et al.*, 2005
Venezuela (North)		*S. metallicum* E[b]	Natural	Limitation	Basáñez *et al.*, 2000; Grillet *et al.*, 1994
Venezuela and Brazil (Amazonian focus)		*S. guianense* s.l.	Natural	Limitation	Basáñez *et al.*, 1995
	S. incrustatum / *S. limbatum*		Natural	Initial facilitation	Grillet *et al.*, 2008
	S. oyapockense / *S. roraimense*		Natural	Initial facilitation	Grillet *et al.*, 2008
Britain	*S. ornatum* s.l.		Natural	N/A	Reid, 1994
		S. lineatum	Experimental	N/A	Reid, 1994

Notes: [a] According to Reid (1994);
[b] letters/names following species are cytoforms;
[c] species of the *S. damnosum* complex are also natural hosts of *O. ochengi*; *S. ornatum* s.l. is a natural vector of *O. lienalis*;
[d] input microfilariae can be microfilariae/mg of skin or microfilariae ingested/fly; output larvae can be exo-peritrophic and thoracic microfilariae or infective, L3 larvae.

the blood meal (Ramos *et al.*, 1994) and constituting a type-1 PM (Lehane, 1997). The PM is composed of proteins, glycoproteins and chitin in a proteoglycan matrix, and among its main functional roles are: 1) partition of the mid-gut lumen into physiologically meaningful compartments, such as separation of the ingested blood from the mid-gut epithelium, with the PM delimiting the gut lumen into a wholly enclosed endo-peritrophic space and an outer ecto-peritrophic space; 2) regulation of digestion and of the passage of molecules between different mid-gut compartments; 3) protection of the mid-gut cells from mechanical abrasion by blood components (e.g., sharp-edged haemoglobin-like crystals) and 4) defence against infection by pathogens and parasites (Lehane, 1997; Ramos *et al.*, 1993, 1994; Shao *et al.*, 2001).

In the context of *Onchocerca–Simulium* interactions, the several molecular, biochemical and physical properties of the PM, determining its rate of synthesis, composition, structure, thickness and degradation time, can be related to the ability of microfilariae to survive and migrate successfully towards the thoracic muscles. In blackflies, the PM starts forming within minutes of blood ingestion, with an initially rapid secretion of PM material from the mid-gut epithelium. This secretory phase is followed by a period of organisation, maximum thickness being achieved between 6 and 12 h post-engorgement (PE) depending on species. The rate of secretion, the level of organisation into distinct (or not) laminae, the resulting morphological appearance, and the final thickness of the PM appear to be species specific (Reid and Lehane, 1984). It has been suggested that only those microfilariae that penetrate the PM during the initial secretion period, before it has condensed and hardened into a distinct structure, or that find themselves adjacent to very thin areas within the matrix, may reach successfully the haemocoel of the blackfly en route to the thorax (Ramos *et al.*, 1994; Reid and Lehane, 1984). Those that do not traverse the abdominal epithelium in time, share the fate of the blood meal and are eventually digested, not contributing to transmission (Lewis, 1953).

11.1.2.4. Vector survival

Lewis (1953) and Omar and Garms (1977) described, respectively, for *S. damnosum* and *S. metallicum*, that in those flies that ingested large numbers of microfilariae, the excessive number of parasites interrupted the formation of the PM and disrupted the normal process of blood digestion, rapidly reaching many organs other than the stomach thus invading and injuring, among others, the foregut, the hindgut, the Malpighian tubules and the haemocoel. This generalised infection led to the death of the insect within a few hours PE. Given the importance that vector survival, until completion of the extrinsic incubation period of the parasite and beyond, has in the transmission of vector-borne infections

(Macdonald, 1957), Basáñez *et al.* (1996) compared mortality rates and expectation of infective life among blackfly species with (*S. ochraceum* s.l.) and without (*S. damnosum* s.l., *S. guianense* s.l.,) well-developed cibarial armatures. In all three species there was evidence of senescence (mortality rates increased with time PE, a proxy for fly's age), and of vector survival being adversely affected by increasing microfilarial load. However, the proportion of flies surviving beyond the maturation period of *O. volvulus* within the fly was higher in armed than in unarmed flies for a given microfilarial intake, suggesting that the cibarial armature affords a certain degree of protection against the damage that high numbers of ingested parasites may cause to the simuliids.

11.1.3. Aims and objectives of this review

Having summarised the various stages in the *Onchocerca–Simulium* interaction, we now proceed to review the methods that have been used and the results that have been obtained when the population consequences of such interaction have been investigated. As population regulation requires the operation of density-dependent processes, we focus on the identification and quantification of these, and their incorporation into the life cycle of *O. volvulus* so that their epidemiological end evolutionary implications can be discussed. We examine available evidence of local adaptation in *Onchocerca–Simulium* complexes and of the parasite responding to selective pressures by exploiting the interaction to maximise its transmission. Finally, we examine the consequences of these processes for the control of human onchocerciasis.

11.2. METHODS

11.2.1. A statistical description of *Onchocerca–Simulium* interactions

The quantitative relationships between consecutive *Onchocerca* stages within *Simulium* have been investigated by fitting appropriate statistical models to parasite density data obtained from series of fly-feeding experiments. The results suggest that many processes involved in the parasite–vector interaction are density dependent, requiring the use of non-linear regression methods or appropriate transformation of variables for linear analysis. In this section we describe briefly the statistical methods used, referring the reader to appropriate literature for detailed explanations that are beyond the scope of this paper.

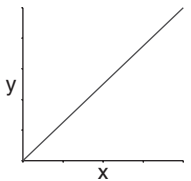
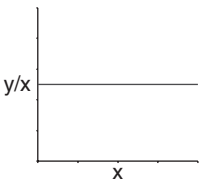
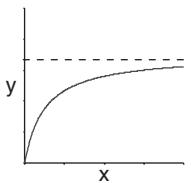
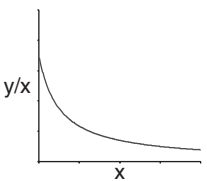
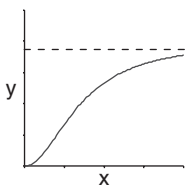
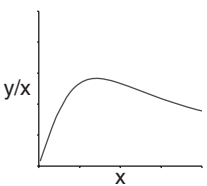
11.2.1.1. Density dependence

In the context of *Onchocerca–Simulium* interactions a biological process is density dependent (non-linear) when the per capita rate at which such process occurs (e.g., microfilarial intake for a given microfilaridermia; fraction of microfilariae damaged by the cibarial armature for a given microfilarial intake; development of microfilariae to the infective stage for a given microfilarial load, etc.) decreases or increases with parasite density. If the rate remains constant it is said to be density independent. In the literature of filariasis–mosquito interactions, the concepts of *limitation*, *facilitation* and *proportionality* have been introduced to describe, respectively, the operation of negative density dependence, positive density dependence and density independence (Pichon, 1974). The per capita rate of successful passage or development to the L3 stage has been referred to as *parasite yield* (Pichon *et al.*, 1974). Table 11.2 (adapted from Sinden *et al.*, 2007) illustrates the possible functional relationships between two consecutive parasite stages within the vector and their corresponding per capita rates or probabilities of success.

11.2.1.2. Parasite distributions and statistical consequences

The distribution of microfilariae in the human host's skin, of ingested microfilariae per fly, and of thoracic and infective larvae among vectors are all highly over-dispersed (i.e., parasites are not randomly distributed, with most hosts or vectors harbouring low parasite loads and few hosts or flies being heavily infected). The statistical study of the relationships between *Onchocerca* stages necessitates, therefore, the use of transformations in an attempt to normalise the distribution of the variables (square root and logarithmic transformations are among the most commonly used, see Basáñez *et al.*, 1994) for application of least squares regression methods using the means in groups of flies. Maximum likelihood estimation with over-dispersed distribution of residuals is a powerful method that permits use of individual fly data (Subramanian *et al.*, 1998). One of the over-dispersed distributions most frequently used is the negative binomial, as it has proven to fit satisfactorily the number of *Onchocerca* larvae per fly among a range of vector species (Cheke *et al.*, 1982 and Renz, 1987 for *S. damnosum* s.l.; Demanou *et al.*, 2003 for *S. squamosum* B; Wetten *et al.*, 2007 for *S. exiguum* s.l., and Grillet *et al.*, 2008 for *S. oyapockense* s.l. and *S. incrustatum*). For measures of central tendency, authors tend to report the arithmetic mean, the median, the geometric mean of Williams (Williams, 1937) and if the variance is stabilised, to perform parametric statistics using log-transformed data and report confidence intervals of geometric means (Kirkwood and Sterne, 2003). The issue of which constant to add to parasite counts before taking their logarithm so that uninfected flies can be included in the analyses depends on the frequency of very low counts (when adding 1

TABLE 11.2 Possible functional forms to describe the relationships between two parasite developmental stages in the *Onchocerca-Simulium* interaction

Functional form	Behaviour	Shape of the relationship between y and x^a	Parasite yield
Linear	Proportionality (no density dependence)		
Non-linear, saturating	Limitation (negative density dependence)		
Non-linear, sigmoid	Initial facilitation and subsequent limitation (positive and negative density dependence)		

Notes:[a] y represents density of the output variable and x density of the input variable in arbitrary units. Examples of input variables are: microfilariae in the skin (microfilariae per mg or per snip), or microfilariae ingested per fly; examples of output variables are successful microfilariae or L3 larvae per fly (adapted from Sinden *et al.*, 2007).

would be inappropriate), and has been discussed by Anscombe (1948) and Soumbey-Alley *et al.* (2004), among others.

Since the explanatory variables when studying *Onchocerca–Simulium* interactions tend to be random variables themselves (e.g., microfilaridermia per milligram of skin or per skin snip; number of microfilariae ingested per fly, etc.) and not truly independent variables, the problem of accounting for measurement error in the explanatory variable is commonly encountered. Measurement error may arise, among other causes, because of the fact we are essentially sampling from a distribution, and because there may be observer's error and inter-observer variation. The presence of measurement error may attenuate relationships, making them more strongly non-linear (accentuating limitation) than they would otherwise be if we had knowledge of the true value of the explanatory variable in question (Carroll *et al.*, 1995). The papers by Basáñez *et al.* (1994), Demanou *et al.* (2003), Soumbey-Alley *et al.* (2004) and Wetten *et al.* (2007) describe various approaches to the problem of measurement error, with the paper by Soumbey-Alley and co-workers (2004) focusing on the method of *instrumental variables*, and that of Demanou and co-workers (2003) on the method of estimating the *reliability ratio*.

The negative binomial distribution (NBD) also provides a useful method for relating the proportion of individuals infected in the population sample (be this of flies or humans) to the mean parasite load via an over-dispersion parameter (best known in the parasitological literature as the k parameter), which may itself be a function of the mean intensity of infection. Such a relationship has been used to describe the proportion of flies with ingested microfilariae that have been fed on given microfilaridermias as well as the proportion of infective flies resulting from such feeds, allowing for inter-specific, inter-cytoform, and intra-specific comparisons of vector competence for varying parasite densities (Basáñez *et al.*, 1994, 1995; Demanou *et al.*, 2003; Grillet *et al.*, 2008; Wetten *et al.*, 2007).

11.2.2. Mathematical models of *Onchocerca–Simulium* population biology and implications for the control of human onchocerciasis

The influence of different *Onchocerca–Simulium* combinations on the population dynamics and population genetics of the parasite has been investigated using a range of deterministic mathematical models. Systems of ordinary (Basáñez and Ricárdez-Esquinca, 2001; Basáñez *et al.*, 2007) and partial (Filipe *et al.*, 2005) differential equations have been developed to describe mathematically the life cycle of *O. volvulus* within the human host and different *Simulium* species. Churcher *et al.* (2005) have explored

the effect of parasite over-dispersion on the introduction and persistence of the infection in models for West African savannah *O. volvulus-S. damnosum* s.s. Where possible the models have been calibrated using parameters estimated by applying the statistical methods summarised in the previous section. Parameters not measured from experimental studies for ethical reasons (e.g., parasite establishment rates within humans) were estimated by fitting models to data relating exposure to blackfly bites with endemic microfilarial load from a range of geographical locations (Basáñez *et al.*, 2002). These deterministic models have been extended to incorporate genetic heterogeneity within the parasite population in order to investigate how the parasite may evolve under various selective pressures, specifically those exerted by widespread use of the microfilaricidal drug ivermectin (Churcher and Basáñez, 2008a,b).

11.3. RESULTS

11.3.1. Relationship between the availability of microfilariae in the skin and microfilarial intake by the fly

In some species a proportional (e.g., *S. ochraceum* s.l. and *S. guianense* s.l.) or nearly proportional (*S. damnosum* s.l.) relationship between the numbers of microfilariae ingested per fly and the numbers per milligram of skin has been reported (Basáñez *et al.*, 1994), whereas in others (e.g., *S. oyapockense* s.l. and *S. incrustatum*), microfilarial intake is negatively density dependent (Grillet *et al.*, 2008) (Fig. 11.3A–D). Demanou *et al.* (2003) compared the intake of various species of the *S. damnosum* complex and concluded that this was, in part, species specific and generally higher among savannah species (*S. damnosum* s.s./*S. sirbanum*) than forest (*S. squamosum/S. yahense*) or forest-savannah mosaic (*S. soubrense/S. sanctipauli*) species given similar skin burdens. There were also differences within species (*S. squamosum* B exhibiting intakes closer to those of savannah flies than *S. squamosum* A or C). These authors argued that in addition to simuliid-specific factors, differences in intakes could also be related to differences in the depth of dermal microfilarial distribution between parasite strains (Bain *et al.*, 1986; Vuong *et al.*, 1988), or the severity of skin disease (lichenification) associated with heavy microfilaridermia, particularly in forest *Onchocerca–Simulium* combinations (Duke, 1962). In contrast to the means of microfilarial intake varying markedly among simuliid species, the prevalence versus intensity relationships of flies with ingested microfilariae show greater similarity among species (Basáñez *et al.*, 1994; Demanou *et al.*, 2003; Grillet *et al.*, 2008), with the proportion of flies having ingested parasites increasing rapidly with mean microfilarial load or intake reaching nearly 100% for high

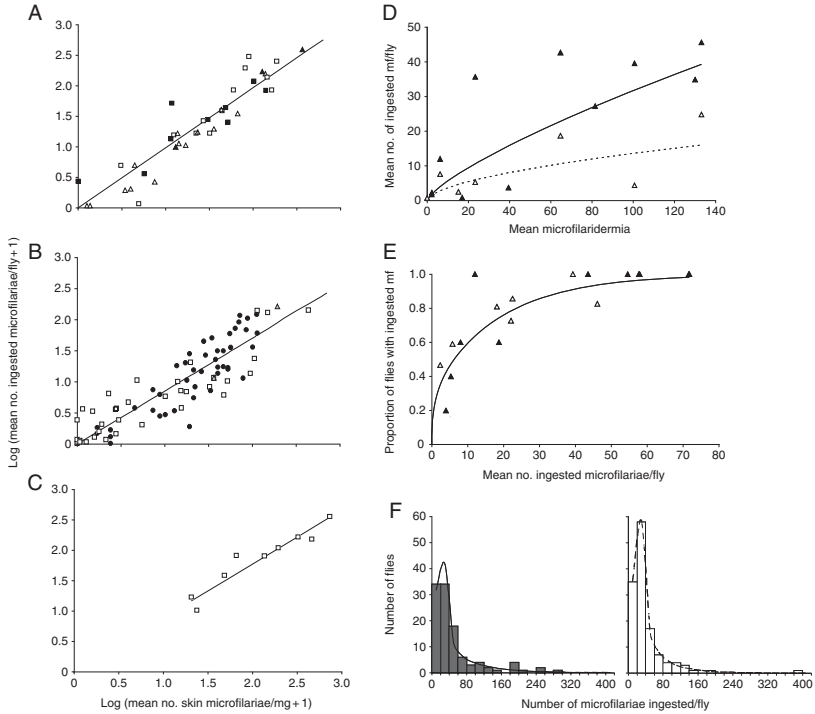


FIGURE 11.3 Ingestion of *O. volvulus* microfilariae by a range of simuliid vectors. The relationship between the number of microfilariae in the skin and the number of microfilariae ingested per fly in (A) *S. ochraceum* s.l. from Guatemala and Mexico; (B) *S. damnosum* s.l. from West African savannah; (C) *S. guianense* s.l., and (D) *S. oyapockense* s.l. (closed triangles) and *S. incrustatum* (open triangles) from the Amazonian focus. (E) The proportion of flies that ingested microfilariae as a function of mean microfilarial intake (markers as in (D)) with fitted function deriving from the negative binomial distribution of ingested microfilariae per fly that is depicted in (F). In (F) dark bars are for *S. oyapockense* s.l. ($k = 0.25$) and white bars for *S. incrustatum* ($k = 0.31$). In (A) open squares are from Campbell *et al.* (1980), closed squares from Collins *et al.* (1977), closed triangles from De Leon and Duke (1966) (Guatemala), and open triangles represent data obtained by Mario Alberto Rodríguez, Concepción Guadalupe Flores-Díaz, and Marco Alecio Sandoval (Mexico) for *S. ochraceum* s.l.; in (B) open squares are from Boussinesq (1991), closed circles are from data presented in Basáñez *et al.* (1995) and Soumbey-Alley *et al.* (2004), and grey triangles are from Philippon (1977) for savannah *S. damnosum* s.l.; (C) open squares are from Basáñez *et al.* (1994) for *S. guianense* s.l. in the Amazonian focus; (D–F) are redrawn from data presented by Grillet *et al.* (2008).

microfilaridermias (Fig. 11.3E). In those species studied, the number of microfilariae in the blood meal among flies fed on microfilarial carriers followed an over-dispersed distribution empirically well described by the NBD (Fig. 11.3F).

11.3.2. The survival of ingested microfilariae and that of the vectors

The proportion of microfilariae damaged by the cibarial armature has been found to decrease with microfilarial intake and to be species specific; Fig. 11.4 illustrates the differences in the proportion of ingested microfilariae left unscathed between *S. ochraceum* s.l. (ranging from 0 for low intakes to 0.6 for high intakes; Fig. 11.4A), *S. oyapockense* s.l. (from 0 to 0.4; Fig. 11.4B), and *S. incrustatum* (from 0 to 0.8; Fig. 11.4C).

Basáñez *et al*. (1996) estimated that the life expectancy and, particularly, the infective life expectancy of groups of simuliids fed on microfilarial carriers and kept in captivity was both fly's age dependent and dependent on mean microfilarial intake. The decrease of life expectancy with increasing ingested burden was more pronounced in blackflies lacking a well-developed cibarial armature than in 'armed' vectors (Fig. 11.5A and 11.5B). Survivorship of wild-caught flies with sharp armatures depends, however, not only on microfilarial load but also on the age-structure of the biting fly population (Fig. 11.5C and 11.5D).

11.3.3. The establishment and development of *Onchocerca* within the thorax of *Simulium*

11.3.3.1. Passage through the peritrophic matrix

The passage of microfilariae through the peritrophic matrix towards the ecto-peritrophic space, and through the abdominal epithelium into the haemocoel and towards the thoracic muscles has been studied both in terms of the dynamics of parasite numbers in the various fly compartments with time post-feeding (Lewis, 1953 and Laurence, 1966 for *S. damnosum* s.l.), and in terms of density-dependent relationships between parasite numbers in the consecutive compartments at given times after the blood meal (Philippon and Bain, 1972 and Soumbey-Alley *et al*., 2004 for *S. damnosum* s.l.; Bain *et al*., 1974 for *S. ochraceum* s.l.). Demanou *et al*. (2003) investigated both aspects in *S. squamosum* B. Microfilariae can be detected in the ecto-peritrophic space as soon as 2–10 min and in the thorax as soon as 20–30 min PE, the numbers in the thorax increasing according to an S-shape curve, and those in the blood meal steadily decreasing (Laurence, 1966). The numbers of 'successful' microfilariae (those escaping imprisonment by the PM, including ecto-peritrophic and haemocoelic microfilariae) plateau between 6 and 8 h PE (Demanou *et al*., 2003), and by 10–12 h PE most of the migration to the thorax has taken place. Jerwood *et al*. (1984) modelled the migration of *O. volvulus* microfilariae within *S. damnosum* s.l. using a simple compartmental process with different transition rates between compartments (blood meal, abdominal haemocoel, thorax), and estimated an average

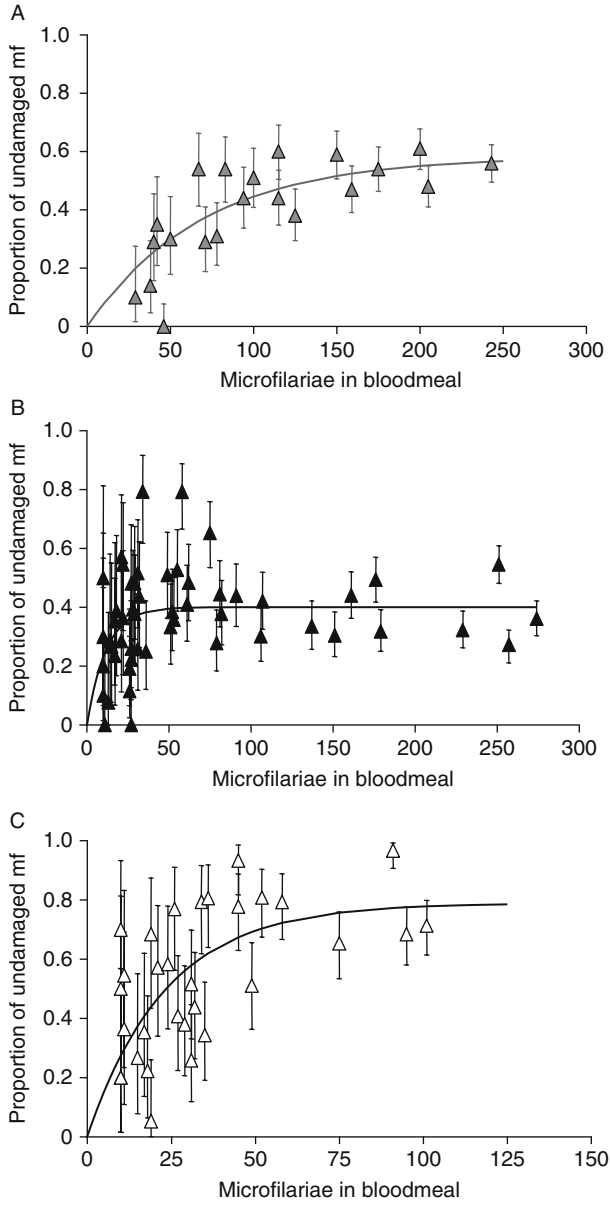


FIGURE 11.4 Proportion of ingested microfilariae left unscathed by the cibarial armature. (A) *S. ochraceum* s.l. from Guatemala (data represented by grey triangles from Bain *et al.*, 1974), (B) *S. oyapockense* s.l. and (C) *S. incrustatum* from the Amazonian focus (data represented, respectively, by black and white triangles, from Grillet *et al.*, 2008). A saturating function described by Basáñez *et al.* (1995) was fitted to the data by maximum likelihood, indicating that the number of microfilariae (mf) lacerated at this

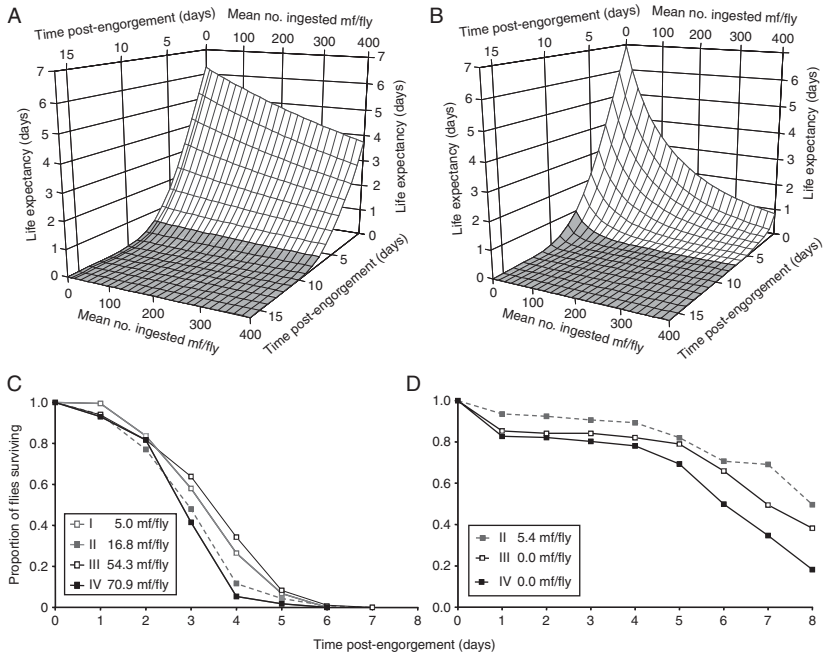


FIGURE 11.5 Effect of *O. volvulus* on the survival of *Simulium.* The (modelled) life expectancy (in days) as a function of both time post-engorgement (PE) (measuring fly's age) and mean microfilarial (mf) intake (measuring parasite load) in (A) simuliids with a well-developed cibarial armature (*S. ochraceum* s.l. from Guatemala), and (B) simuliids with a poorly developed armature (*S. damnosum* s.l. from West Africa and *S. guianense* s.l. from the Amazonian focus). The grey-shaded area represents the expectation of infective life assuming an extrinsic incubation period of 7 days (see Fig. 11.6B). Life expectancy and infective life expectancy decrease faster with increasing microfilarial load in simuliids not protected from parasite-induced damage by a well-developed cibarial armature. In both groups of flies the mortality rate increases with age (original analyses in Basáñez *et al.*, 1996*)*. The proportion of surviving *S. oyapockense* s.l. with time PE is plotted for flies fed on microfilarial carriers before (C), and 6 months after ivermectin treatment (D), in the Venezuelan part of the Amazonian focus. When fed on the same subjects (I–IV), fly survivorship is better after microfilaridermia has been substantially reduced, but differences in the age-structure of the fly population 6 months apart may have also played a part (compare survivorship in both groups for ~5 mf/fly).

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stage of the Onchocerca–Simulium interaction within armed vectors is both density dependent and species specific, with the percent of undamaged parasites ranging from 0% to 60% in *S. ochraceum*, 0% to 40% in *S. oyapockense* and 0% to 80% in *S. incrustatum*. Error bars are exact 95% confidence intervals. The various scales in the x-axis reflect species-specific differences in microfilarial intakes.

duration of 1.6 h in the blood meal, and of 3.1 h in the haemocoel, confirming that those parasites that migrate out of the PM early are the most likely to reach the thorax and proceed towards further development. Fig. 11.6A depicts the observed and predicted proportion of microfilariae in each compartment with time PE.

11.3.3.2. Successful microfilariae and L3 larvae versus available microfilariae per fly

When plotting the numbers and/or proportions of 'successful' microfilariae (those that have migrated out of the blood meal), or the numbers of L3 larvae that develop against microfilarial load in the skin area upon which groups of flies have been fed or against mean microfilarial intake, three distinct patterns emerge (Table 11.1): 1) African savannah flies of the *S. damnosum* complex (Fig. 11.7A–C) and the remaining Latin American simuliid vectors with poorly developed cibarial armatures exhibit limitation (*S. exiguum* s.l. (Fig. 11.8C and 11.D), *S. metallicum* s.l. (Fig. 11.8E), *S. guianense* s.l. (Fig. 11.8F)); 2) vectors with well-developed cibarial armatures exhibit initial facilitation (*S. ochraceum* s.l. (Fig. 11.8A and 11.B), *S. oyapockense* s.l. and *S. incrustatum* (Fig. 11.8G)), and 3) African forest and forest-savannah mosaic flies exhibit proportionality (*S. leonense* (Fig. 11.7D), *S. squamosum* B (Fig. 11.7E, F)), with numbers of successful microfilariae 10-fold higher than in savannah flies (however, how exactly this impacts L3 yield or vector survival needs further investigation). In forest foci, larval loads per naturally infected fly are also higher and the annual transmission potential (ATP, the yearly number of L3 that a person would potentially receive if maximally exposed to blackfly bites) can easily exceed levels that would be intolerable in the savannah because of their association with high blindness prevalence (Duke, 1968). The 'milder' forest strain might also be less virulent to *Simulium*, or may elicit a weaker immune response within the vectors.

Bain *et al*. (1976) proposed that parasite density-dependent changes in the thickness and rate of formation of the PM may explain the limitation observed in the number of ingested microfilariae that gain access to the thoracic muscles in savannah species of *S. damnosum* s.l. Other possible explanations include the activation of insect defences such as lectin-like molecules that interfere with microfilarial migration in filarial-culicid systems by acting at the level of the mid-gut epithelium (Phiri and Ham, 1990). At the level of the blackfly's haemocoel, Hagen and Kläger (2001) observed rapid (and species-specific) killing of *Onchocerca* microfilariae that was mediated by haemocytes and involved increased apoptosis levels in the microfilariae. Humoral (haemolymph) responses identified in simuliids include chiefly two types of inducible immune molecules, the serine proteases and the carbohydrate-binding lectins (Hagen *et al*., 1995, 1997a), with prophenoloxidase also being up-regulated in *Onchocerca*-infected

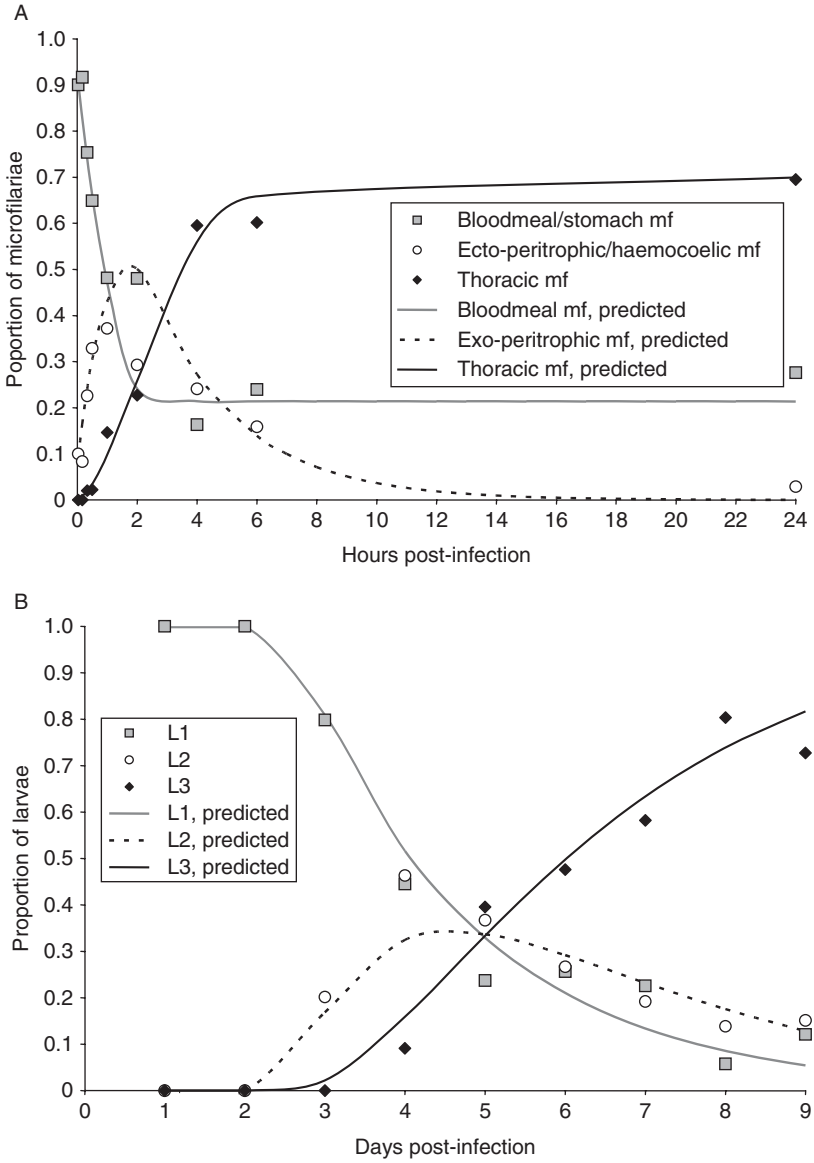

FIGURE 11.6 Compartmental models. (A) Migration of *O. volvulus* microfilariae from the blood meal to the thoracic muscles in *S. damnosum* s.l. in West Africa (data from Laurence, 1966; model by Jerwood *et al.*, 1984), and (B) development of *O. volvulus* larvae from the L1 to the L3 stage within the thoracic muscles of *S. exiguum* s.l. in Ecuador (data collated by Wetten *et al.*, 2007). The average durations in each compartment (A) or developmental stage (B) are the reciprocal of the transition rates estimated by the models. Microfilariae (mf) leave the blood meal on average 1.6 h and the abdomen

Simulium damnosum s.l. (Hagen *et al.*, 1997b)—although melanisation of larvae does not take place. More recently, other types of inducible peptides with anti-bacterial and anti-parasitic activity have been identified in blackfly's haemolymph; similarities of the immune response kinetics between bacterial and filarial infections suggest that intracellular *Wolbachia* bacteria, released from microfilariae, could be responsible for the anti-bacterial response (Kläger *et al.*, 2002). Although it is tempting to suggest that these *Simulium* immune responses may be involved in the phenomenon of limitation, more research is needed to elucidate whether their strength against *Onchocerca* larvae (see also Ham, 1986; Ham *et al.*, 1990, 1994) correlates positively with the density of the inoculum, or with parasite strain. The West African forest strain has lower *Wolbachia* levels (Higazi *et al.*, 2005) and may elicit weaker anti-bacterial responses within simuliids, helping to explain the higher larval loads observed in *Onchocerca–Simulium* forest combinations.

Whereas in some *Onchocerca–Simulium* combinations (savannah species of the *S. damnosum* complex; *S. ochraceum* s.l., *S. guianense* s.l.) the numbers of exo-peritrophic (ecto-peritrophic plus haemocoelic) and thoracic microfilariae per fly are nearly 1:1 predictors of resultant numbers of L3 larvae (Basáñez *et al.*, 1995; Collins *et al.*, 1977; Soumbey-Alley *et al.*, 2004), in others thoracic establishment of early larval stages is not necessarily linked to competent vector status. In southern Venezuela, anthropophagic populations of *S. exiguum* s.l. outside endemic areas are able to ingest *O. volvulus* microfilariae and allow them to reach and penetrate the thoracic muscle fibres. However, larval development does not proceed beyond the L1 stage, failing at the first moulting, with internal parasite structures becoming vacuolated and disintegrated (Basáñez *et al.*, 2000). These *S. exiguum* s.l. populations are, therefore, refractory to *O. volvulus*. By contrast, *S. exiguum* s.l. is the only known vector in Colombia (Tidwell *et al.*, 1980), and the Cayapa cytoform of the *exiguum* complex is a very efficient vector in Ecuador (Shelley and Arzube, 1985), though the numbers of thoracic larvae that establish surpass the numbers of L3 larvae

(ecto-peritrophic space and abdominal haemocoel) 3.1 h after being ingested. After 6 h PE there is no much change in the numbers of microfilariae reaching the thorax. Once in the thorax, larvae remain as L1 larvae for 4.8 days and as L2 larvae for 1.9 days on average; L3 larvae can be seen as early as 4–5 days in the thorax of S. exiguum as a result of natural larval progression. Models were fitted by multinomial maximum likelihood. In (A) grey squares and solid line represent, respectively, observed and modelled proportions of hourly total microfilariae that are in the blood meal (stomach), open circles and broken line correspond to abdominal microfilariae that have left the stomach, and black diamonds and solid line to microfilariae that have reached the thorax. In (B) the same markers and lines have been used to represent, respectively, data and model fits for the proportions of total daily larvae that are in the L1, L2, and L3 stages.

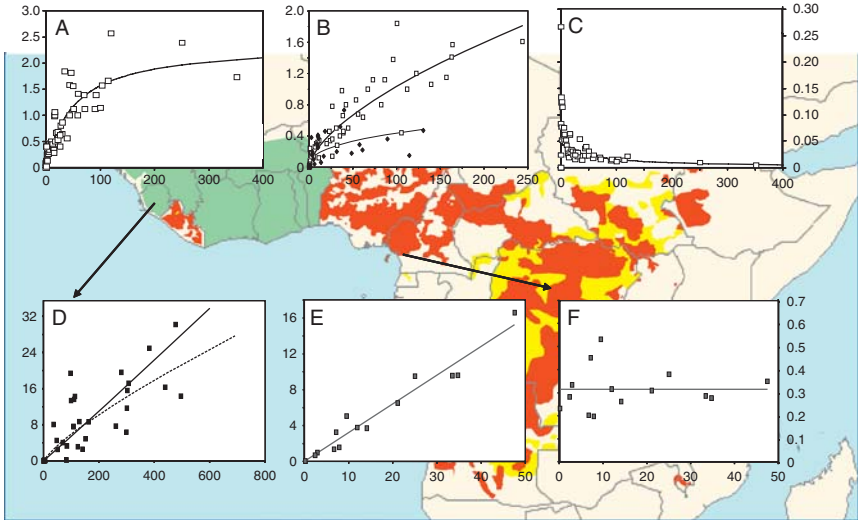

FIGURE 11.7 Relationships between successful and ingested or skin microfilariae in *S. damnosum* s.l. Upper panel (A–C) corresponds to Sudan-savannah species, and lower panel (D–F) to forest/forest-savannah mosaic species. In (A) the y-axis represents mean number of exo-peritrophic and thoracic microfilariae per fly in *S. damnosum* s.s./*S. sirbanum* (open squares) analysed together and the x-axis is mean microfilarial intake (data and saturating model as described in Basáñez *et al.*, 1995); in (B) *S. damnosum* s.s. (open squares) and *S. sirbanum* (closed diamonds) are analysed separately (data and model as described in Soumbey-Alley *et al.*, 2004) and the x-axis is mean microfilaridermia per skin snip. In (C), parasite yield (on the right y-axis) decreases monotonically with increasing microfilarial intake (x-axis), indicating limitation of microfilarial uptake in the Sudan-savannah species. (D) The relationship between exo-peritrophic microfilariae per fly (y-axis) and skin microfilariae per snip (x-axis) for *S. leonense* (forest-savannah mosaic, Sierra Leone), indicating a linearly proportional relationship between the two variables (the dotted non-linear fit is not statistically significantly better than the straight line), with an approximately constant microfilarial uptake of 4–5%; data and analysis (black squares and fitted lines) from Soumbey-Alley *et al.* (2004). (E, F) *S. squamosum* B, from the Sanaga valley in Cameroon (forest) exhibits proportionality, with (E) representing the number of haemocoelic microfilariae (y-axis) versus the number of ingested microfilariae (x-axis) per fly, and (F) the resultant (y-axis) parasite yield (an approximately constant 30% microfilarial uptake); data and analysis (grey squares and fitted lines) from Demanou *et al.* (2003).

produced (Collins *et al.*, 1995). This suggests the operation of yet unknown factors within the fly's thorax regulating parasite density, competition among larvae for space and resources, etc. In other cytoforms of the complex (Aguarico, Bucay and Quevedo) parasite yield has been shown to be significantly lower after adjusting for the limitation effect of negative density dependence (Wetten *et al.*, 2007). We have developed a simple compartmental model to estimate the transition rates and average

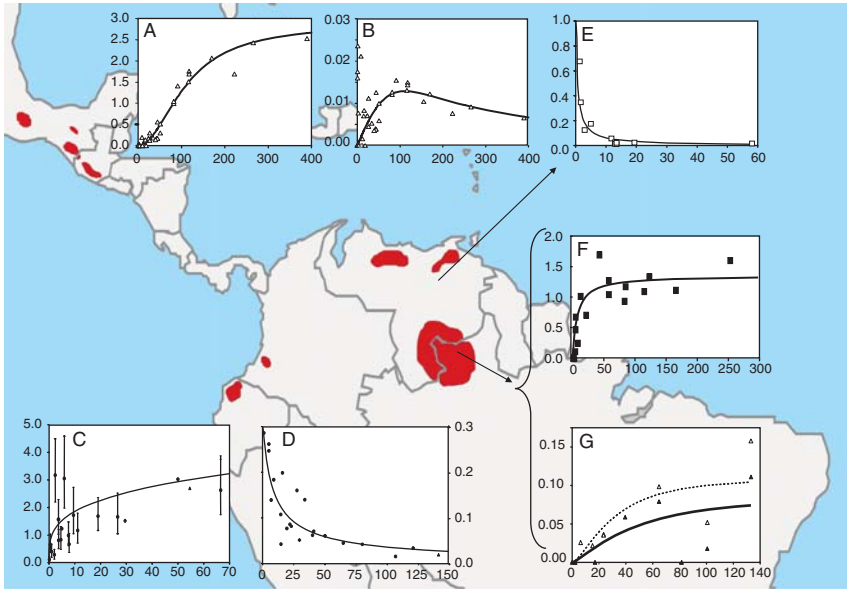

FIGURE 11.8 Relationships between successful and ingested or skin microfilariae in Latin American vector complexes. (A) and (B) represent, respectively, the number of successful microfilariae or L3 larvae per fly and the corresponding parasite yield (y-axes) versus mean microfilarial intake (x-axes) for *S. ochraceum* s.l. in Guatemala (data as grey open triangles collated by Basáñez *et al.*, 1995, with sigmoid grey line fit indicating initial facilitation. Parasite yield initially increases and subsequently decreases with mean number of microfilariae ingested per fly). (C) The number of L3 larvae per fly (left y-axis) and (D) parasite yield (right y-axis) versus microfilaridermia (per mg of skin, on the x-axes) for the Cayapa form of *S. exiguum* s.l. in Ecuador (data as black symbols collated by Wetten *et al.*, 2007, with circles from Collins *et al.*, 1995, diamond from Tidwell *et al.*, 1980, triangle from Shelley and Arzube, 1985, and non-linear black line fit indicating limitation). Parasite yield decreases monotonically with microfilarial density. (E) Parasite yield (y-axis) versus microfilaridermia (x-axis) in *S. metallicum* s.l. from northern Venezuela, data collated by Basáñez *et al.* (2000) demonstrating limitation. (F) The number of successful microfilariae or L3 larvae per fly (y-axis) versus microfilarial intake (x-axis) for *S. guianense* s.l. from the Amazonian focus, with data (black squares) and saturating model fit (black line) presented by Basáñez *et al.* (1995). (G) The mean number of L3 per fly (y-axis) versus skin microfilarial load (x-axis) for *S. oyapockense* s.l. (black triangles for data and solid line for sigmoid model fit) and *S. incrustatum* (white triangles and broken line) from the Amazonian focus (Grillet *et al.*, 2008). The sigmoid fit in both these (armed) species suggests initial facilitation. (Background map prepared by Gaizka Ormaza.)

duration of each larval stage within the Cayapa form of *S. exiguum* s.l. (Fig. 11.6B). Allowing for an initial delay in the L1 compartment and variable transition rates, the mean duration of *O. volvulus* as an L1 larva is 4.8 days and of an L2 larva is 1.9 days.

In *S. metallicum* s.l. the PM is thin (Lewis and Garnham, 1959) and its rate of formation slow (Omar and Garms, 1977), favouring migration of microfilariae out of the blood meal, and in northern Venezuela, populations of *S. metallicum* s.l. from endemic areas allow thoracic establishment (subject to density-dependent limitation, Fig. 11.8E). Yet, larval development tends to be asynchronic, substantially reducing parasite yield (Basáñez *et al*., 2000; Grillet *et al*., 1994).

11.3.4. Local adaptation of *Onchocerca–Simulium* and its consequences for the spread of human onchocerciasis outside currently endemic areas

The notion of well-adapted *Onchocerca–Simulium* complexes (Duke *et al*., 1966) was based on the observation that, in Cameroon, microfilariae of West African forest parasites developed efficiently in *S. damnosum* from West African forest but poorly or not at all in Sudan-savannah flies. Conversely, the success of Sudan-savannah parasites was high when developing within savannah *S. damnosum* but significantly reduced within forest flies (see also Philippon, 1977). Analysis of a tandemly repeated DNA sequence family present in the genome of *O. volvulus* (O-150) confirmed that West African rainforest and savannah parasite populations are significantly different and that some barrier preventing genetic exchange between these two populations must have developed, the existence of vector–parasite complexes offering a possible explanation (Zimmerman *et al*., 1994). However, the situation is likely to be more complex, as there was better development of the forest parasite strain in Guinea-savannah and forest-savannah mosaic-dwelling simuliids (Duke *et al*., 1966). Changes in the epidemiological landscape of West Africa in the last two to three decades, partly due to the Onchocerciasis Control Programme (OCP), may have disrupted such complexes (Toé *et al*., 1997). Interestingly, non-significant differences were found between Guatemalan and North Venezuelan *O. volvulus* populations in their ability to infect local simuliids (*S. ochraceum*, *S. metallicum*, *S. callidum* and *S. haematopotum* in Guatemala, and *S. metallicum* in Venezuela) (Duke, 1970; Takaoka *et al*., 1986a). This was interpreted as close genetic proximity between these two parasite populations (Takaoka *et al*., 1986b). Interestingly, West African forest parasites developed somewhat better in Guatemalan simuliids than Sudan-savannah parasites (De Leon and Duke, 1966). This may be in contrast with reported results that American populations of *O. volvulus* are more closely related to those of West African savannah than forest (Zimmerman *et al*., 1994), or may be due to strain-specific antigenic stimulation of the *Simulium*'s immune system as mentioned above.

In an evolutionary context, the hypothesis that host–parasite interactions are expected to result in geographical patterns of adaptation in which parasites are better able to infect their local host populations (Lively and Jokela, 1996), has been confirmed by experimental infection studies across a wide range of host–parasite systems (Failloux *et al*., 1995; Lively, 1989; Parker, 1985). Laurence and Pester (1967) described the relatively rapid adaptation of the filarial worm *Brugia patei* to a new mosquito host, *Aedes togoi*, in the laboratory and discussed the implications of this finding in explaining present distribution of filariases.

If mutually compatible *Onchocerca–Simulium* complexes exist within each main endemic area, the question arises as to what is the true potential for onchocerciasis to spread towards new regions where infected carriers may settle and high densities of anthropophagic simuliids occur. Evidence of locally acquired onchocercal infections, or potential for this to take place outside original endemic areas has been documented in Brazil and Ecuador (Charalambous *et al*., 1997; Guderian and Shelley, 1992; Maia-Herzog *et al*., 1999). In Venezuela, Basáñez *et al*. (2000) investigated the compatibility between sympatric and allopatric combinations of *O. volvulus*-*Simulium* in the northern onchocerciasis focus (where flies of *S. metallicum* cytospecies E were fed on microfilarial carriers from the northern and Amazonian foci), and in a densely populated locality of Amazonas outside the main Amazonian focus (where *S. oyapockense* s.l. and *S. exiguum* s.l. were fed on the aforementioned microfilarial carriers). For the homologous northern *O. volvulus*-*S. metallicum* combination, parasite yield was 45% in contrast to 1% for the heterologous, Amazonian *O. volvulus*-*S. metallicum* infection. This was significantly lower than the parasite yield (4–10%) that would have been expected in the sympatric combination after allowing for density-dependent limitation of L3 output in *S. metallicum*. The anthropophagic population of *S. exiguum* s.l. from southern Venezuela allowed no larval development beyond the L1 stage of either northern or Amazonian parasites (see Section 11.3.3.2). The parasite yield of Amazonian *O. volvulus* in *S. oyapockense* s.l. biting humans in the capital of the Amazonas State was about 1%, in agreement with figures ranging from 0.02% to 1.23% recorded for the sympatric combination within the Amazonian focus (Grillet *et al*., 2008). By contrast, no L3 development of the northern parasite was observed in southern *S. oyapockense*. These results, together with considerations of typical microfilarial loads in humans in the northern and Amazonian foci, poorly developed (*S. metallicum*) or well-developed (*S. oyapockense*) cibaria in the blackflies (Shelley *et al*., 1987), parasite-induced vector mortality (high in *S. metallicum*; Omar and Garms, 1977), and fly biting rates (of the order of thousands per person per day for *S. oyapockense* s.l. in the Amazonian lowlands; Grillet *et al*., 2001), suggest a lower potential for onchocerciasis to spread between the northern and Amazonian endemic areas of

Venezuela than that between Amazonian hyperendemic, untreated locations and settlements outside this focus with high densities of anthropophagic *S. oyapockense* s.l. Table 11.3 presents a (non-exhaustive) summary of experimental infections conducted to study the cross-compatibility of *Onchocerca–Simulium* combinations in African and Latin American endemic areas.

11.4. IMPLICATIONS FOR OUR UNDERSTANDING OF THE POPULATION AND EVOLUTIONARY BIOLOGY OF *O. VOLVULUS* AND THE CONTROL OF HUMAN ONCHOCERCIASIS

In this section we use mathematical models to coalesce and interpret the results of the previous sections and discuss how in-depth knowledge of vector–parasite interactions informs current understanding of the transmission dynamics of the infection and helps identify optimal control strategies. Fig. 11.9 presents the life cycle of *O. volvulus*, illustrating at various points the density-dependent processes that may be operating. Churcher *et al*. (2005) have combined density dependence and overdispersed parasite frequency distributions in savannah *Onchocerca–Simulium* complexes, and Churcher *et al*. (2006) have investigated the separate and combined effects of density dependence on rates of parasite re-infection after treatment.

11.4.1. Consequences of density dependence in *Onchocerca–Simulium* interactions for efforts to achieve local elimination of *O. volvulus*

The aim of the Onchocerciasis Elimination Program for the Americas (OEPA) is to eliminate the *O. volvulus* reservoir in the Americas and not just the public health burden (Richards *et al*., 2001). There are also focal areas within Africa where parasite elimination is deemed possible. The feasibility of this goal may well depend on the particular vector complex or complexes that are present in the areas targeted for elimination, particularly where programme objectives rely almost entirely on reducing microfilaridermia by mass administration of ivermectin. In principle, local parasite elimination can be achieved by lowering parasite density to such an extent that: (1) the remaining adult male and female worms do not inhabit the same host or females cannot be fertilised, thus not producing microfilariae (interrupting transmission from humans to vectors), or (2) most, if not all, of the few microfilariae ingested by armed simuliids (in areas where these prevail) are damaged, thus not proceeding towards

TABLE 11.3 Summary of cross-experimental infections to assess local adaptation of *Onchocerca-Simulium* complexes

Country (Type)[a]	*O. volvulus-Simulium* combination	Subject (Locality)	Microfilariae per fly	L3 larvae per fly	Parasite yield	Reference
Cameroon (S)	West Africa Forest *O. volvulus* & *Simulium*[b]	I (Bolo & Sanaga)	3.74–11.0	2.25–5.12	0.47–0.86	Duke *et al.*, 1966
		II (Bolo)	0.94–8.60	0.66–5.60	0.65–0.86	
Bioko (S)	W. A. Forest *O. volvulus* & *Simulium*[c]	I (Tiburones)	3.61	1.61	0.45	Duke *et al.*, 1966
Sierra Leone (S)	W. A. Forest *O. volvulus* & *Simulium*[d]	I (Magburaka)	5.93	3.74	0.63	Duke *et al.*, 1966
Cameroon Burkina Faso (S)	Sudan savannah *O. volvulus* & *Simulium*[e]	III (Mayo Boki & Volta Blanche)	1.10–4.05	0.40–1.88	0.36–0.47	Duke *et al.*, 1966
		IV (M. Boki)	16.5	2.53	0.15	
Cameroon Burkina Faso (A)	W. A. Forest *O. volvulus*-S. savannah *Simulium*	I (M. Boki & Grand Capitaine)	6.38–7.77	0–0.10	0–0.01	Duke *et al.*, 1966
		I (V. Blanche)	15.1	0	0	
		II (M. Boki)	2.18	0.19	0.09	
Cameroon (A)	S. savannah *O. volvulus*-W. A. Forest *Simulium*	III (Bolo)	1.0–3.87	0–0.09	0–0.02	Duke *et al.*, 1966
		IV (Bolo)	18.5	0.14	0.01	
Sierra Leone (A)	S. savannah *O. volvulus*-W. A. Forest *Simulium*	III (Magburaka)	1.47	0.13	0.09	Duke *et al.*, 1966
Guatemala (S)	Guatemalan *O. volvulus*-*S. ochraceum*	I	9.0–390	0.19–2.53	0.02–0.01	De Leon & Duke, 1966
		II	170	2.07	0.01	
Guatemala (S)	Guatemalan *O. volvulus*-*S. metallicum*	I	6.0	0.15	0.03	De Leon & Duke, 1966
		I	190	Intake fatal	0	
		II	5.0	0.06	0.01	

Guatemala (A)	W. A. Forest *O. volvulus-S. ochraceum*	III	14.0	0.24	0.02	De Leon & Duke, 1966
Guatemala (A)	W. A. Forest *O. volvulus-S. metallicum*	III	16.0	0.05	0.003	De Leon & Duke, 1966
Guatemala (A)	S. savannah *O. volvulus-S. ochraceum*	IV	1.4	0	0	De Leon & Duke, 1966
Guatemala (A)	S. savannah *O. volvulus-S. metallicum*	IV	0.9	0	0	De Leon & Duke, 1966
Northern Venezuela (S)	Venezuelan *O. volvulus-S. metallicum*	VIII (Altamira) IX (Altamira) X (Altamira)	2.78 9.85 19.9	0.58 0.86 0.66	0.21 0.09 0.03	Duke, 1970
N. Venezuela (S)	Venezuelan *O. volvulus-S. exiguum*	VIII (El Loro) X (El Loro)	1.63 14.9	0.09 0.04	0.06 0.003	Duke, 1970
N. Venezuela (A)	W. A. Forest *O. volvulus-S. metallicum*	VI (Altamira, Carabobo)	4.66	0	0	Duke, 1970
N. Venezuela (A)	S. savannah *O. volvulus-S. metallicum*	VII (Altamira, Carabobo)	24.0	0.01	0.0004	Duke, 1970
N. Venezuela (A)	W. A. Forest *O. volvulus-S. exiguum*	VI (El Loro, Aragua)	1.55	0	0	Duke, 1970
N. Venezuela (A)	S. savannah *O. volvulus-S. exiguum*	VII (El Loro, Aragua)	2.14	0	0	Duke, 1970
Guatemala (S)	Guatemalan *O. volvulus-S. metallicum*	I (Chimaltenango)	0.80	0.05	0.06	Takaoka et *al.*, 1986a

(*continued*)

Table 11.3 (*continued*)

Country (Type)[a]	*O. volvulus-Simulium* combination	Subject (Locality)	Microfilariae per fly	L3 larvae per fly	Parasite yield	Reference
Guatemala (A)	Venezuelan *O. volvulus*-Guatemalan *S. metallicum*	II (Chimaltenango)	3.00	0.40	0.13	Takaoka *et al.*, 1986a
N. Venezuela (S)	Venezuelan *O. volvulus*-*S. metallicum*	II (Rio Chiquito, Monagas)	7.31	0.47	0.06	Takaoka *et al.*, 1986b
N. Venezuela (A)	Guatemalan *O. volvulus*-Venezuelan *S. metallicum*	I (Rio Chiquito, Monagas)	1.95	0.08	0.04	Takaoka *et al.*, 1986b
N. Venezuela (S)	N. Venezuelan *O. volvulus*-*S. metallicum*	Ia (Carrasposo, Anzoátegui)	2.13	0.95	0.45	Basáñez *et al.*, 2000
N. Venezuela (A)	Amazonian *O. volvulus*-N. Venezuelan *S. metallicum*	IIb (Carrasposo, Anzoátegui)	10.9	0.11	0.01	Basáñez *et al.*, 2000

[a] (S) Sympatric (homologous) combination, (A) Allopatric (heterologous) combination;
[b] Possibly *S. squamosum* at Sanaga
[c] *S. yahense* Bioko form
[d] *S. leonense*
[e] *S. damnosum s.s./S. sirbanum.*

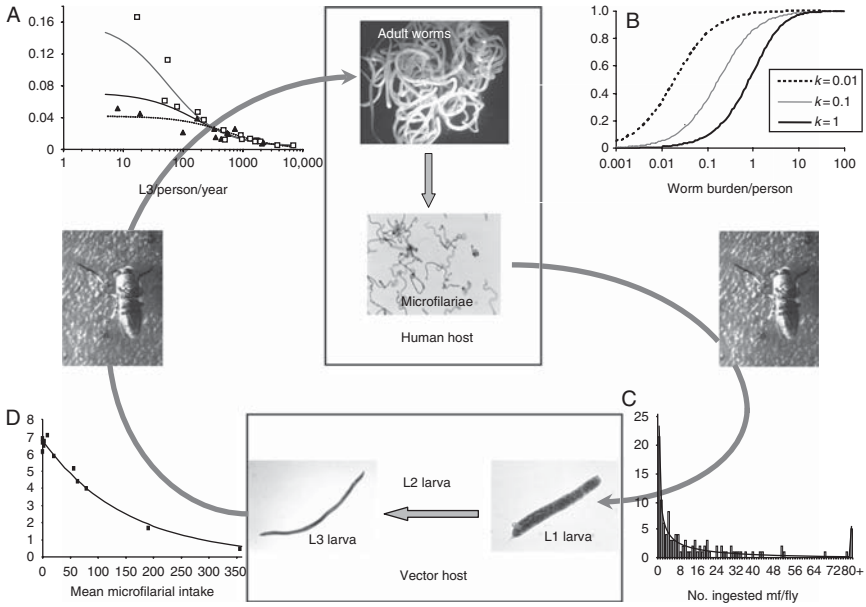

FIGURE 11.9 Life cycle of *Onchocerca volvulus* and density-dependent processes. (A) Transmission of L3 larvae via blackfly bites results in a per capita parasite establishment rate that decreases with the rate of human exposure to infective larvae (negative density dependence), with data and model fits redrawn from those presented by Basáñez *et al.* (2002); open squares for West Africa and black triangles for Meso America, with grey, black and dotted lines representing a range of plausible fits. Established worms become adult males and females within subcutaneous nodules. (B) The probability that a female worm is fertilised (positive density dependence) increases both with mean worm burden and degree of over-dispersion, with a decreasing value of parameter k (from the negative binomial distribution, NBD) indicating stronger parasite clumping. Given a mean worm burden, the mating probability increases faster for $k = 0.01$ (dotted line), than for $k = 0.1$ (grey line) or $k = 1$ (solid black line). Fertilised females produce microfilariae (mf) that migrate to the skin, being ingested with a blood meal (Fig. 11.2). (C) The distribution of the number of microfilariae per fly is also clumped and well-fitted by the NBD (adapted from Demanou *et al.* (2003) with $k = 0.4$). Microfilariae establish within the thoracic muscles (Figs. 11.2 and 11.6A) with per capita rates of establishment (uptake) that can decrease, initially increase or remain constant (i.e., can exhibit negative (Figs. 11.7C, 11.8D and 11.8E), positive (Fig. 11.8B), or no density dependence (Fig. 11.7F). (D) Life expectancy of the vector may be adversely affected by increasing microfilarial intake, another form of negative density dependence (data jointly analysed for *S. damnosum* and *S. guianense*, black markers and fitted line, from those presented by Basáñez *et al.*, 1996). Photo credits: adult worms from the TDR Image Library (WHO/TDR/OCP; image ID: 9303242); microfilariae emerged from incubated skin snips by Carlos Botto, Amazonian Centre for Research and Control of Tropical Diseases (CAICET); L1 and L3 larvae within *Simulium guianense* by María-Gloria Basáñez; biting female blackfly (*S. guianense* from the Amazonian focus) by Carlos Ayesta, Faculty of Sciences, Universidad Central de Venezuela.

larval development (interrupting transmission from vectors to humans). Therefore, both these transmission-blocking strategies rely on the operation of positive density dependence, the former affecting the probability that a female worm is mated (Fig. 11.9B), and the latter the probability that an ingested microfilaria develops into an L3 larva in simuliids with well-developed cibarial armatures and hence initial facilitation. The mating probability can in theory be estimated using information on mean worm burden per host, the ratio of male to female worms, the frequency distribution of worms per host and the sexual system of the parasites (May, 1977; May and Woolhouse, 1993), assumed to be polygamous in *O. volvulus* (Schulz-Key and Karam, 1986). Using recent estimates derived from fitting a mathematical model to microfilarial data from Guatemala, pre-ivermectin values for mean worm burden and corresponding (NBD) over-dispersion parameter were, respectively, of the order of 47 worms per person and k was 0.35 (Bottomley *et al.*, 2008). With these figures, and a balanced sex ratio, the initial mating probability is very close to 1 (all female worms would be mated). In practice the mating probability of a strongly polygamous and clumped parasite is likely to remain high during most of the control programme until the worm burden is substantially reduced, or the parasite sex ratio markedly altered (e.g., if male worms were significantly more sensitive to the drug), or female insemination rates were disrupted (Cupp and Cupp, 2005). By the same token, initial facilitation in armed blackflies is likely to have an impact only when the vector biting rate is very close to the threshold necessary to maintain endemic transmission; for biting rates well above this threshold in the absence of vector control, parasite densities below which transmission would be blocked (transmission breakpoints) will be extremely low. These concepts are illustrated by plotting the effective reproduction ratio (R_e) of *Onchocerca* against mean microfilaridermia (Fig. 11.10), although it must be stressed that the results presented here are only intended to provide qualitative insight rather than accurate quantitative predictions (as they are derived using a deterministic model and most likely overestimate the worms' mating probability). R_e is defined as the average number of adult female progeny produced during the reproductive life span of an adult female worm given a mean worm burden, and, therefore, is subject to density dependence (unlike the basic reproduction ratio, or R_0). The parasite intensity below which the operation of positive density dependence restricts population growth to such an extent that $R_e < 1$ (each female worm is unable to replace itself) is the breakpoint density (Macdonald, 1965). The effective reproduction ratio of *O. volvulus* in an area where *S. ochraceum* s.l. is the most important vector species is presented in Fig. 11.10A for different annual biting rates (ABR). Larger values of the vector biting rate reduce the breakpoint density, making *O. volvulus* harder to eliminate (Duerr *et al.*, 2005). Different

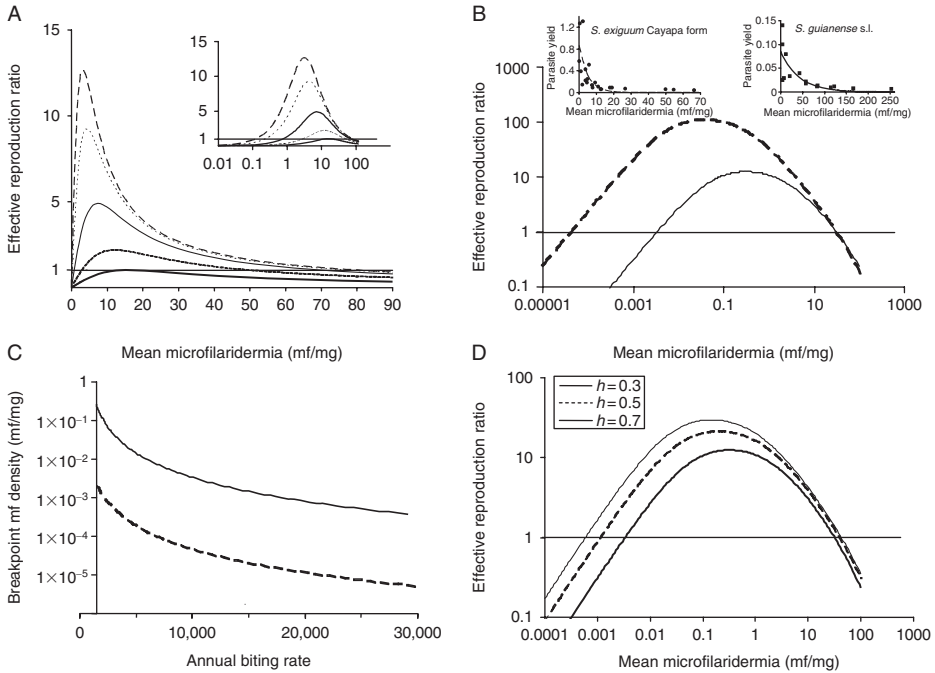

FIGURE 11.10 Transmission breakpoints in human onchocerciasis. The effective reproduction ratio (R_e, with density dependence) is plotted against mean microfilaridermia in (A), (B) and (D). A value of $R_e = 1$ indicates equilibrium (each female worm replaces itself, represented by the points where the curves cross the horizontal solid line), with endemic equilibria corresponding to those lying on the right-hand side of maximum R_e, and unstable equilibria (breakpoint densities) to those lying on the left-hand side. The lower the breakpoint densities are, the harder it will be to achieve local elimination of onchocerciasis. (A) R_e values for increasing annual biting rates (ABR = number of flies landing to bite as recorded in Mexico and Guatemala and collated by Basáñez *et al.*, 2002) for *S. ochraceum* s.l. (8,800 flies person^{-1} year^{-1}, thick solid,

Onchocerca–Simulium complexes have different types and severities of positive and negative density-dependent regulatory mechanisms which will alter the parasite breakpoint densities (Fig. 11.10B and 11.10C). Higher proportion of blood meals taken on humans by the local vector population will lower breakpoint microfilaridermia (Fig. 11.10D). However, care should be taken when interpreting breakpoint densities as some residual transmission may be maintained even when $R_e < 1$, and parasite burden may oscillate around the breakpoint density, taking many years to be truly eliminated (Gambhir and Michael, 2008). Increases in the vector population during this period, or seasonal changes in vector density (Grillet *et al.*, 2001; Vieira *et al.*, 2005) may allow the parasite to re-establish itself in the host population. In general, under the current strategy of mass distribution of ivermectin without additional measures of vector control, the risk of re-infection may be higher than previously assumed (Duerr *et al.*, 2006).

11.4.2. The evolution and spread of ivermectin resistance in *O. volvulus* populations

Current reliance of onchocerciasis control programmes on mass distribution of ivermectin, and reports of elevated microfilarial loads in treated individuals and populations (Awadzi *et al.*, 2004a,b; Osei-Atweneboana *et al.*, 2007) raise the prospect that drug resistance may become a public health concern (but see Cupp *et al.*, 2007; Mackenzie, 2007; and Remme *et al.*, 2007 for a debate as to whether the above mentioned observations

bottom line (El Jardín); 33,127, thin dashed line (Nueva América); 92,585, thin solid line (Los Tarrales); 301,065, dotted line (Los Andes); 550,559, thick dashed line (Santa Isabel). The ABR at El Jardín is very close to the threshold biting rate assuming that all blood meals are taken on humans. As vector density increases, endemic microfilarial loads increase and transmission breakpoint densities decrease (see inset for details), highlighting that vector control could play a crucial role in aiding parasite elimination. (B) For two vector species with negative density dependence, and assuming equal endemic microfilarial load (~30 microfilariae (mf)/mg), the stronger limitation quantified for *S. exiguum* s.l. (upper left inset and dashed line) in comparison to that for *S. guianense* s.l. (upper right inset and solid line) reduces breakpoint microfilaridermia (ABR = 10,000; 1 in 3 blood meals are on human hosts). (C) The larger the endemic ABR value, the smaller the breakpoint microfilaridermia (solid line, *S. guianense*; dashed line, *S. exiguum*; anthropophagy as in (B)), highlighting once more the role that vector control could have in elimination efforts. (D) The more anthropophagic a vector population, the harder to achieve local elimination, with the proportion of blood meals taken on humans (h) equal to 0.3 (thick solid, bottom line), 0.5 (dashed line), and 0.7 (thin solid, top line) for *S. guianense* s.l. with ABR = 10,000. Predicted endemic microfilaridermia values range from ~30 ($h = 0.3$) to ~40 ($h = 0.7$) mf/mg).

may be due to causes other than drug resistance). If resistance alleles were present within populations of *O. volvulus* (it is expected that they would initially be rare), their rate of spread would be influenced by the density, competence and biting behaviour of the local simuliid vectors.

Predicting how drug resistance will spread through different *Onchocerca–Simulium* complexes requires a fuller understanding of the genetics of ivermectin resistance in human filariases, as well as of the processes (immunological or otherwise) that regulate parasite establishment within the human host and how these will be affected by chemotherapy. Some onchocerciasis mathematical models assume that L3 larvae provide the antigenic stimulus for protective immune responses and that immunological memory is relatively short, with the per capita rate of parasite establishment decreasing with increasing ATP (Fig. 11.9A) (Basáñez *et al*., 2002; Duerr *et al*., 2008). As mass drug administration progresses, microfilaridermia in treated hosts and overall transmission would initially decrease. Decreased ATP levels would result in increased parasite establishment rates, giving an advantage to any resistant parasites that may be present (whose microfilariae would have had a greater chance of reaching the L3 stage). As resistant parasites replace the drug-sensitive worm population, areas with higher vector biting rates and/or highly competent vectors will also recover higher ATPs, with higher net numbers of parasites and skin microfilariae. Therefore, drug resistance might become more evident in areas with higher vector biting rates and endemic transmission potentials in the absence of vector control (Fig. 11.11B). Other models have assumed facilitated parasite establishment mediated by the number of adult worms already present (Duerr *et al*., 2003). Depending on whether or not repeated ivermectin treatment has macrofilaricidal effects (Cupp and Cupp, 2005), establishment rates of resistant parasites may be initially reduced as drug-sensitive parasites are affected. Ivermectin acts by both killing *O. volvulus* microfilariae (microfilaricidal effect) and inhibiting their production by female worms (embryostatic effect) for several weeks (Basáñez *et al*., 2008). Therefore, repeated ivermectin treatment would result in rapid and substantial changes of putative resistance allele frequency in populations of skin microfilariae and L3 larvae in vectors (Fig. 11.11C).

Treatment would reduce the impact of negative density-dependent processes restricting parasite abundance in humans and vectors. The relaxation of these regulatory processes will increase the contribution of resistant parasites to the subsequent generation (Churcher and Basáñez, 2008a,b). Vectors that exert strong limitation on microfilarial uptake will tend to give resistance alleles greater selective advantage after chemotherapy. This is because most drug-sensitive parasites will be within those untreated hosts who have high microfilaridermia. Conversely, drug-resistant parasites will be harboured by treated hosts, whose initial

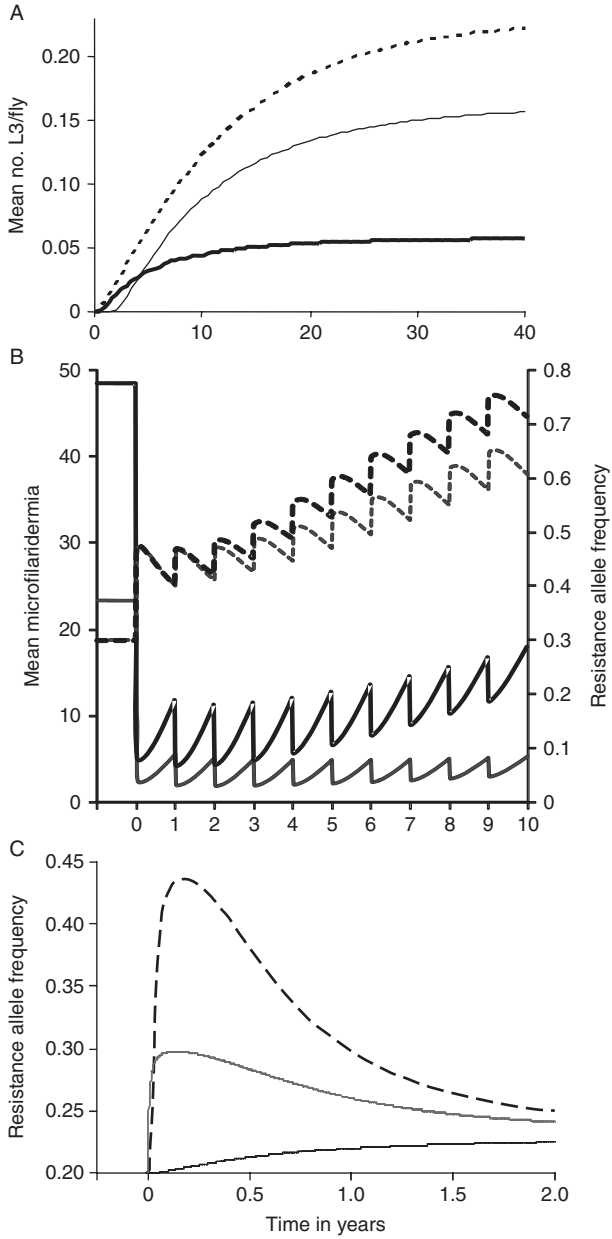

FIGURE 11.11 Spread of anthelmintic resistance in *O. volvulus*. (A) Predicted mean infective larval load in Sudan-savannah *Onchocerca–Simulium* combinations by models that ignore (dotted line) or incorporate (solid lines) parasite frequency distribution plus density dependence in vectors and humans. The mean-based model overestimates infective larval load and does not capture appropriately initial dynamics at the time of

microfilaridermia will be lower, increasing parasite yield. Also, stronger limitation will lower transmission thresholds (Fig. 11.10B and 11.10C), making local elimination more difficult. Vectors with strong facilitation may, on the contrary, restrict the spread of drug-resistant parasites, particularly at low resistance allele frequencies.

The number of bites received by hosts may depend on their age and sex (Filipe *et al.*, 2005). A heterogeneous biting rate may cause hosts to acquire new infections at different rates, which could result in the resistance allele frequency varying between hosts (Churcher and Basáñez, 2008b). At low allele frequencies parasite genetic differentiation between hosts enhances the spread of recessive alleles, by increasing the proportion of offspring that will be homozygous. Under the assumption that ivermectin resistance is recessive, this will lead to a faster spread of resistant parasites (Schwab *et al.*, 2007). The genetics of ivermectin resistance in *O. volvulus*, however, is likely to be more complex and possibly polygenic.

Should drug resistance develop in one area of the control programme, it is likely to spread geographically relatively quickly. Blackflies are known to migrate long distances, both in search of suitable breeding sites and through wind-assisted movement (Boakye *et al.*, 1998). Results from the small number of genetic studies that have been undertaken in *O. volvulus* suggest that there is limited intra-specific structure

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introduction. The models with moderate ($k = 0.1$, thin solid line) and strong ($k = 0.01$, thick solid line) parasite aggregation predict a lower larval load and enhance the probability of introduction and persistence (redrawn from Churcher *et al.*, 2005). (B) The influence of vector biting rate on the detection (by increasing microfilaridermia, left-hand y-axis, solid lines) and spread (resistance allele frequency, right-hand y-axis, dashed lines) of ivermectin resistance as predicted by mathematical models that introduce genetic structure into *O. volvulus* populations with respect to drug susceptibility. The model (Churcher and Basáñez, 2008a) has been parameterised for *Onchocerca–Simulium* combinations from the Sudan-savannah bioclimatic zone. The annual biting rate is 15,000 (black lines) or 2,000 (grey lines). Ivermectin coverage is at 80% annually for 10 years. For illustrative purposes only, drug resistance is conferred by a single, autosomal recessive allele which protects the parasite from the microfilaricidal and embryostatic effects of ivermectin. Initial resistance allele frequency is 0.3. (C) The impact of ivermectin treatment on mean (within treated and untreated hosts) resistance allele frequency of different *O. volvulus* life-stages: adult worms within humans (solid black line); microfilariae in skin (grey line), and L3 within vectors (dashed line). The temporal dynamics are shown following a single round of ivermectin to 80% of the human population. Assumptions are as in (B) excepting initial resistance allele frequency which equals 0.2 (redrawn from Churcher and Basáñez, 2008a). Sampling infective larvae in vector populations may be an appropriate strategy to monitor changes in resistance allele frequency following chemotherapy.

(Morales-Hojas *et al.*, 2007). This would facilitate the flow of resistance alleles between different African populations.

11.5. DISCUSSION AND FUTURE RESEARCH DIRECTIONS

The centre for adaptive radiation of the genus *Onchocerca* (mainly parasites of ungulate mammals) is the African continent (Bain, 1981), with *O. volvulus* belonging to a small, highly specialised group that evolved from parasites of African savannah bovids (Krueger *et al.*, 2007). The African savannah and forest strains of *O. volvulus* are genetically distinct (Zimmerman *et al.*, 1994), transmitted on the whole by different simuliid species and correlate well, respectively, with severe and mild ocular disease (Higazi *et al.*, 2005; Zimmerman *et al.*, 1992). The greater compatibility between sympatric as opposed to allopatric parasite–vector combinations suggests the operation of local adaptation, which would provide indirect evidence of co-evolution (Woolhouse *et al.*, 2002). In practice, demonstrating co-evolution in *Onchocerca–Simulium* would involve testing for reciprocal adaptive genetic change, and documenting fitness benefits for either parasite or vector associated with the trait in question (Poulin, 1998). However, lack of animal models for *O. volvulus* and difficulties in colonising *Simulium* in the laboratory have hampered understanding of their formal genetics and hindered opportunities for tightly controlled experimental approaches.

It has been proposed that natural selection would favour parasites that can manipulate their vectors to enhance their transmission (Hurd, 2003). The factors that contribute to parasite transmission success are encapsulated in the formulation of its basic reproduction ratio, R_0. With respect to the *Onchocerca–Simulium* interactions, crucial factors are the amount of contact between vectors and humans (when parasites are transmitted to and from blackflies), the probability that an ingested microfilaria reaches the infective stage (vector competence), and the probability that infected flies survive completion of *O. volvulus* extrinsic incubation period and beyond (vector survivorship and infective life expectancy). Therefore, parasite strategies may include increasing the contact rate (to our knowledge, an area of research not much pursued in blackflies), enhancing the input of microfilariae or the output of L3 larvae, augmenting vector competence, and lengthening vector longevity. Vectors, in turn, may mount anti-parasitic defences or develop parasite-avoidance strategies, while the parasites may develop the ability to evade or suppress host defences (Hurd, 2003; Koella, 1999).

In Section 11.3.3.2 work confirming the operation of innate and acquired resistance to filarial infection in blackflies was summarised. As a possible counteracting mechanism, Kläger *et al.* (1999) found a

cysteine protease inhibitor, onchocystatin, which was present in female adults, skin microfilariae and excretory-secretory microfilarial products of the bovine parasite *O. ochengi* Bwangamoi (the closest relative of *O. volvulus*). Co-injection of onchocystatin and *O. ochengi* microfilariae into the thorax of surrogate host *Simulium ornatum* Meigen s.l. significantly enhanced parasite establishment rates within 24 h post-infection, suggesting a possible role of onchocystatin in the evasion by the parasite of the simuliid's immune response. Further research could focus on confirming the role of onchocystatin in the evasion of immune responses by members of the *S. damnosum* complex, the natural vectors of *O. ochengi* (Omar *et al*., 1979; Wahl *et al*., 1998).

Another strategy for increasing parasite transmission success would be for the parasite to deplete vector's reproductive output. This would increase nutrient reserves available for the parasite while increasing vector longevity, as decreased oviposition rates would reduce vector mortality risks associated with egg laying. Although evidence for *O. volvulus*-induced reduction of simuliid fecundity is scarce, we would predict that in unarmed blackflies, in which the proportion of ingested microfilariae that reach the thorax is larger and competition for resources possibly stronger, negative effects of infection on vector reproduction would be more pronounced than in vectors with a well-developed armature. In support of this conjecture are the results of Ham and Banya (1984), who infected *S. lineatum* Meigen (a species lacking cibarial projections) and *S. ornatum* s.l. (a species with toothed cibarium) with varying microfilarial numbers of *O. lienalis* Stiles (an *Onchocerca* of cattle naturally transmitted by *S. ornatum*). In (unarmed) *S. lineatum*, and infecting the flies *per os*, fecundity was reduced by 21 to 76% of that of uninfected controls, with the magnitude of the effect depending on parasite concentration in the blood meal. A reduction by 21% was observed in (armed) *S. ornatum* but only when flies were fed on concentrations of microfilariae as large as 69,000 per ml. *S. lineatum* also showed a depression of oviposition rates when infected by *O. lienalis* larvae. Interestingly, the feeding rates in those groups of flies offered infected blood were reduced in several instances, suggesting the possibility of parasite avoidance. Intra-thoracic injection (which circumvents any earlier barrier to successful infection) also decreased fecundity, with reductions for *S. lineatum* depending on inoculum (36% for 10 microfilariae/fly; 54% for 50 microfilariae/fly). By contrast, reductions in *S. ornatum* s.l. were of a lower magnitude, reaching 13% when injected with 20 microfilariae/fly. Subsequently, Renshaw and Hurd (1994a,b) demonstrated that in the system *O. lienalis–S. ornatum*, and when 20 microfilariae were intra-thoracically injected immediately after a blood meal, there was a significant reduction of ovarian vitellin content; inocula lower than 20 did not exert a dose-dependent effect. Extending these experimental approaches to a range of natural *O. volvulus* vectors with well

characterised parasite success rates would be of interest for the elucidation of parasite-induced and density-dependent effects on components of vector fitness.

It is still unknown to what extent *O. volvulus* has adapted to selective pressures exerted by the various constraining and/or facilitating regulatory processes that operate in different vector species, or whether some of these processes have themselves evolved in response to parasite pressure. Besides, in many endemic areas there is more than one simuliid species that can act as a vector for the parasite, and although there may be a predominant species, species composition and abundance can change spatially and temporally (Grillet *et al.*, 2001; Vieira *et al.*, 2005; Vivas-Martínez *et al.*, 1998). Consequently, in a single endemic area selective pressures may operate in opposing directions. In simuliids with cibarial armatures and positive density dependence, increasing microfilarial availability or transmissibility to the flies may be an advantageous strategy for the parasite. Given that female worm fertility is already high (more than 1,000 microfilariae are produced daily per female; Schulz Key, 1990), and that their distribution in the skin is highly clumped (Kershaw *et al.*, 1954), the issue may be one of optimising allocation. One aspect that has received some attention is that of a positive correlation between the distribution of microfilariae and of vector bites along the human host's body, with for instance, microfilaridermia levels of the savannah strain of *O. volvulus* being higher in the lower half of the body (where *S. damnosum* preferentially bites; Renz and Wenk, 1983) and the converse being observed in Mesoamerica (Brandling-Bennett and Darsie, 1983; Kawabata *et al.*, 1980), where *S. ochraceum* frequently attacks the neck and upper torso (Dalmat, 1955).

If we accept the conjecture that the cibarial armature in simuliids is a trait evolved in response to haematophagia and not in response to parasite pressure (Reid, 1994), it may be advantageous for *O. volvulus* to exploit blackfly's salivary secretions, dermal microfilariae being attracted towards the inflicted wound. The paired blackfly comparisons listed in Section 11.1.2.2 seem to conform to expectation and rigorous testing of this hypothesis in a range of armed and unarmed species may be a fruitful research avenue. Even if armed simuliids ingest large numbers of microfilariae, the armature will protect the fly from excessive mortality, so the extrinsic incubation period can be completed and the parasite transmitted (Basáñez *et al.*, 1996). However, the selective advantage of parasite genes which may enhance microfilarial concentration to the site of the wound would be reduced by negative density dependence operating on microfilarial passage out of the stomach, limitation of thoracic development, or increased vector mortality in unarmed simuliids, which all decrease the probability an ingested microfilaria will develop into an L3 at high loads.

The interaction between the different positive (facilitating) and negative (constraining) density-dependent processes acting within the

parasite's life cycle may cause the maximum (overall) level of transmission to occur at intermediate skin microfilarial densities, with the distribution of those densities among and within human hosts being highly over-dispersed. Optimal density will also depend on the local biting rate (s) of the simuliid vector(s). Genetically structured mathematical models combining population dynamics and genetics may provide insights into evolutionary stable strategies for different *Onchocerca–Simulium* complexes. However, before we can implement such models much research is still required on the selective pressures that may operate for the evolution of susceptibility, resistance, virulence, infectivity and transmissibility in this system, the amount of variability present in the parasite's and vectors' genome for potentially relevant traits, and to demonstrate that parasites and simuliids have reciprocal effects on each other's phenotype and genotype (Webster *et al*., 2004). Improving our understanding of the epidemiological and biomedical significance of co-evolution will require combining the phenomenological approaches of population and evolutionary biology with the search for mechanistic explanations underlying the parasitological and entomological patterns described in this paper, and making use of the extensive data that are becoming available on the molecular biology and genetics of parasite-vector interactions (Woolhouse *et al*., 2002).

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CHAPTER 12

Microsporidians as Evolution-Proof Agents of Malaria Control?

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Contents

Abstract

Despite our efforts at malaria control, malaria remains one of our most serious and deadly diseases. The failure of control stems in part from the parasite's intense transmission in many areas and from the emergence and spread of resistance of the malaria parasites and their mosquito vectors against most of the chemicals used to attack them. New methods for control are desperately needed. However, new methods will be useful only if they are effective (i.e., decrease transmission substantially) and evolutionarily sustainable (i.e., evolution-proof, in that they prevent evolution from eroding efficacy). We suggest microsporidian parasites that infect mosquitoes could be potentially effective and sustainable agents for malaria control. They may be effective because they target several epidemiologically important traits: survival of larvae (and thus number of adult mosquitoes), adult longevity, biting rate and the development of malaria within the mosquitoes. Even if each trait is affected only moderately, the intensity of transmission can be reduced considerably. They may be evolution-proof, for the evolutionarily most important trait is juvenile survival, whereas the two epidemiologically most important factors are traits of the adult mosquito: biting rate and longevity.

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Under the intense microsporidian pressure of a control programme, it is likely (if not inevitable) that the larvae evolve to survive microsporidian infection. However, if this larval tolerance to microsporidians is genetically correlated with the adult traits, tolerant mosquitoes may not live as long and bite less frequently than microsporidian-sensitive ones. While such a trade-off has not been measured, combining several studies suggests indirectly a negative genetic correlation between larval tolerance and adult longevity. Therefore, evolution might not undermine control; rather it might increase its effectiveness. While the evolution of resistance may be inevitable, the failure of control need not be.

12.1. INTRODUCTION

Despite the vision of the Bill and Melinda Gates Foundation to eradicate malaria, raised in October 2007 at its Malaria Forum, there is considerable doubt (Roberts and Enserink, 2007; Tanner and de Savigny, 2008) that the available tools can eliminate the 500 million clinical cases and 2 million deaths per year (World Health Organization (WHO), 2005). Some of the problems in controlling malaria are socio-economic: it is difficult to integrate control tools into existing health and social systems, to maintain surveillance strategies with existing health systems, and to synchronise efforts among areas (Tanner and de Savigny, 2008). Other problems are biological. First, the parasite's intense transmission, which in highly endemic areas is two orders of magnitude higher than that of many of our common, more easily controllable diseases such as measles (Smith *et al.*, 2007), makes it very difficult to block transmission. Basic epidemiological models show that measles, for example, can be eliminated from a population if about 90% of children are vaccinated; the equivalent number for malaria in endemic areas is more than 99%. The difficulty in controlling malaria in highly endemic areas is reflected in a recent report by the WHO (2008). While bed nets and drugs cut the malaria burden by as much as half in three countries with low endemicity (Zanzibar, Ethiopia and Rwanda), no such success was seen in the one country with high endemicity (Ghana). Although the report emphasises that the lack of success may be due to the low funding of the control programme, the high transmission may have also contributed to the failure of control. Second, if the best tools available today—artemisinin-based combination therapy, long-lasting insecticide-treated bed nets and indoor insecticide spraying—become more widely used, it is expected (if not inevitable) that their efficacies will decrease as resistance of the malaria parasite to drugs and of the mosquito vector to insecticides spreads.

Malaria control thus needs new tools. Such tools should be (i) effective, so that they can decrease transmission substantially, and (ii) evolutionarily sustainable (i.e., evolution-proof), so that they can prevent (or at least delay) the decrease of efficacy associated with resistance against the control tool.

Unfortunately, while some of our current control tools are effective or aim at being sustainable, few (if any) are both. Insecticide-treated bed nets, for example, are effective because they affect the two parameters with the most impact on malaria transmission (Macdonald, 1957): the mosquito's longevity and (as the insecticides also function as repellents and act as a physical barrier) their biting rate. These aspects will be discussed in more detail below. However, as resistance to the insecticides used on bed nets has already spread to several malarious areas (e.g., Elissa *et al.*, 1993; Hargreaves *et al.*, 2000; Kasap *et al.*, 2000), the fear is that the method is not sustainable (although the level of resistance observed to date has not prevented the nets from working acceptably (Henry *et al.*, 1999)). Artemisinin-based combination therapy was developed in an attempt to delay the emergence and spread of drug resistance. However, theory suggests that using two drugs instead of one delays the spread of resistance only in areas with low transmission (Pongtavorninyo, 2006) and indeed, the first cases of resistance have appeared (Jambou *et al.*, 2005; Sisowath *et al.*, 2005).

Will future control agents be better? If successful vaccines can be developed, they may be effective, but it is unlikely that they will be evolution-proof, as there is strong selection pressure for the parasites to develop escape-mutants, and evolution may even lead to more damaging parasites if the vaccines do not completely block infection (Gandon *et al.*, 2001, 2003). The genetic strategy to drive resistance genes linked to a transposable element through populations is unlikely to be effective (Boëte and Koella, 2002; Koella and Zaghloul, 2008), as they target an inefficient parameter: the density of susceptible mosquitoes. Furthermore, as resistance is likely to be evolutionarily costly, evolution may dissociate the transposable element linked to the resistance gene that the mosquitoes are transformed with, making the strategy unsustainable (Marshall and Chou, 2007). An alternative genetic strategy—transforming males to be sterile—may be sustainable, but is unlikely to be effective, as it targets an ineffective parameter (number of adult mosquitoes). Furthermore, density-dependent population regulation at the larval stage may reduce the influence of sterilised males on the population density. An alternative possibility is the suggestion of using entomopathogenic fungi for the evolution-proof control of malaria (Blanford *et al.*, 2005; Thomas and Read, 2007). Such fungi increase the mosquito's mortality, in particular if they are infected by malaria, and decrease their ability to blood-feed late in their life. As mentioned above, longevity and biting rate are critical

parameters of malaria transmission, so that fungi could reduce transmission effectively. As the effects occur late in the mosquitoes' life, evolutionary theory tells us that mutations that would decrease the effects of the fungus to increase the mosquitoes' health late in life are subject to weak selection. Therefore, resistance would appear only very slowly, if at all.

Here we discuss aspects of effective and sustainable control in more detail, illustrate the approach with microsporidians that infect mosquitoes, and suggest that microsporidians are considered as potential agents for malaria control. Most of the experimental work underlying this idea has been done with the microsporidian *Vavraia culicis*, of which the life cycle is described in Box 12.1.

12.2. EFFECTIVE CONTROL

The epidemiology of malaria is described by the Ross-Macdonald equation of malaria transmission (Macdonald, 1957): $R_0 = \frac{ma^2be^{-\mu T}}{r\mu}$, where m is the number of adult female mosquitoes per human, a is the mosquito's

BOX 12.1 Life cycle and biology of *Vavraia culicis*

Microsporidia are obligate intracellular parasites of animals; most of them infect insects. They evolved from a fungus (Keeling *et al.*, 2000) and have then been extremely reduced, for example, by losing their mitochondria. *Vavraia culicis* Weiser is an obligate, intracellular microsporidian parasite of several mosquito species. It is horizontally transmitted when mosquito larvae ingest its spores. After several rounds of replication within a larva, the parasite begins to produce its infectious spores. In some cases, in particular in conditions of food stress or intense infection (Bedhomme *et al.*, 2004), these kill the larva or pupa and are released into the breeding site, thus initiating another round of horizontal transmission. In other cases, larvae survive the infection and develop into adults. There is no transovarial transmission (i.e., the parasite does not penetrate the eggs of infected females; Becnel *et al.*, 2005), but spores can adhere to the surface of the eggs and infect the newborns (Andreadis, 2007).

In experiments where individually reared larvae were exposed to the spores, the next generation of spores first appeared several days after infection and the number of spores then increased more or less exponentially with the duration of infection, irrespectively of the stage of the mosquito.

biting rate (on humans), b is the probability that a mosquito that has bitten an infectious person becomes infected, μ is the daily mortality of the mosquito, T is the parasite's developmental period in the mosquito and r is the recovery rate of humans. A sensitivity analysis of this equation (Bailey and Duppenthaler, 1980), which estimates the efficacies of malaria control based on different methods, shows that adult mortality and biting rate are the two parameters that are most effective at reducing malaria transmission. While, for example, halving mosquito density halves R_0 (as m is a linear parameter), halving biting rate (a quadratic parameter) reduces R_0 by 75% and doubling adult mortality (which occurs as an exponential and a linear term) decreases R_0 by about 80%. It is for this reason that insecticides used for malaria control are usually applied as insecticide-impregnated bed nets (which increase mortality and decrease biting rate) or with indoor insecticide spraying (which kills adults) rather than as larvicides (which reduce the number of mosquitoes).

We suggest that microsporidians, focusing here on *V. culicis*, have the potential for effective malaria control as they affect several of the parameters in R_0, including the epidemiologically most relevant ones.

(i) Mosquito density. Microsporidians kill some of the larvae and pupae (although larval mortality in field conditions is rarely high; Andreadis, 2007). Not surprisingly, the parasite's ability to kill the juveniles increases with the number of spores the larvae are exposed to (Fig. 12.1A). The epidemiologically relevant consequence of killing juveniles is that it reduces the number of adult females. Note that *V. culicis* (and other microsporidians) often have little effect early in infection, but rather kill their hosts as late-stage larvae or pupae. Therefore, density-dependent effects (which could have the paradoxical effect that killing some larvae enhances transmission, for the surviving larvae develop into larger adults that carry more parasites (Lyimo and Koella, 1992) and survive longer (Ameneshewa and Service, 1996) are at most weak.

(ii) Adult longevity. In one experiment, in which larvae were fed under optimal food conditions, microsporidian infection reduced the mean longevity of adults, on average, by 10% (from 19.2 to 17.2 days). Some microsporidian isolates had little to no effect while others reduced longevity to less than 15 days (Fig. 12.1B). In an experiment with a restricted larval diet, microsporidians had a larger impact on adult longevity, reducing it from 18.6 to 13.8 days.

(iii) Infectivity of malaria to mosquitoes. Several microsporidians impede the development of malaria parasites within the mosquito, so that microsporidian-infected mosquitoes are less likely to harbour oocysts and sporozoites, and harbour fewer of them (Bano, 1958; Fox and Weiser, 1959; Gajanana *et al.*, 1979; Hulls, 1971; Schenker

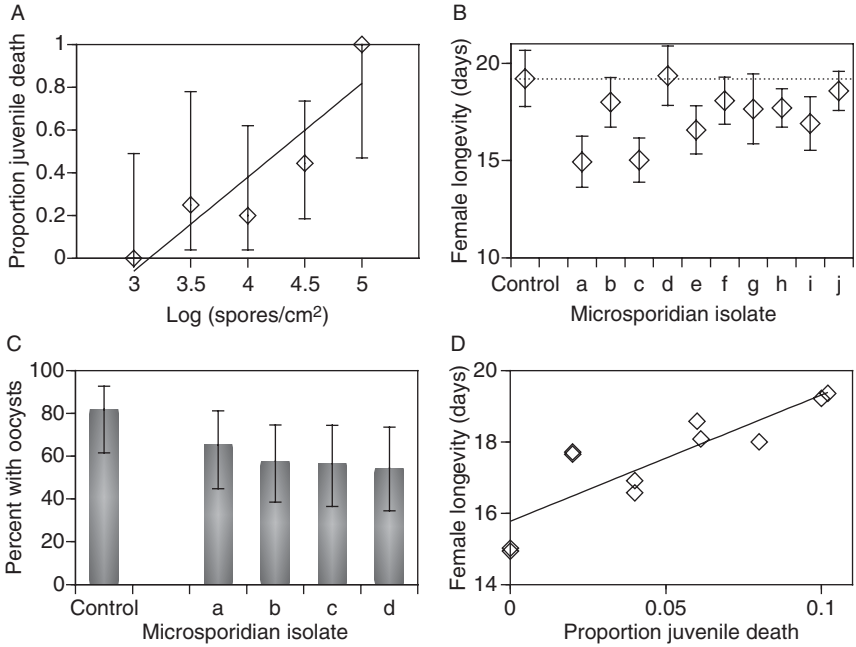

FIGURE 12.1 Effects of microsporidians on selected traits of mosquitoes. (A) Effect of various concentrations of *Vavraia culicis* on the proportion of *Aedes aegypti* dying before emergence (Fellous and Koella, unpublished data). The diamonds show the means within treatments, and the vertical bars show the 95% confidence intervals of proportions. (B) Longevity of adult, female *Anopheles gambiae* infected by one of 10 isolates of *V. culicis* or uninfected (Lorenz and Koella, unpublished data). The diamonds show the mean values, the vertical bars show the standard errors of the means. The dotted line indicates the longevity of the uninfected controls. (C) Success of infection (proportion of A. gambiae with at least one oocyst 10 days after blood feeding) by *P. berghei* in control mosquitoes and mosquitoes infected by one of four isolates of *V. culicis* (Bargielowski and Koella, under review). The bars show the proportions, the vertical bars show the 95% confidence intervals of proportions. (D) Association between the juvenile mortality and the adult longevity of *A. gambiae* infected with one of 10 isolates of *V. culicis* (Lorenz and Koella, unpublished data). The symbols show the means of isolates, the line shows the regression through the means.

et al., 1992). *Vavraia culicis* is no exception. In an experiment with the mouse malaria *Plasmodium berghei* it decreased the percentage of mosquitoes infected with oocysts from 82% to 58% (Fig. 12.1C) and reduced the mean number of oocysts (in malaria-positive mosquitoes) from 21 to 9. Again, isolates differed in their effect, at least in reducing the number of oocysts: the most effective isolate reduced the mean number of oocysts to 4.8 (not shown).

(iv) Biting rate. No data are yet available on the effect of *V. culicis* on the biting rate of mosquitoes. However, *Edhazardia aedis*, the only microsporidian of mosquitoes for which an effect on biting rate has been studied, completely inhibits biting if infection is sufficiently intense (Koella and Agnew, 1997).

It is this targeting of several parameters (including the epidemiologically most relevant ones: biting rate and adult longevity) that could make microsporidians an effective control agent, even if each parameter is affected only moderately. Indeed, if we consider values similar to those observed in our experiments (larval mortality increased by 25%, adult mortality increased by 20% and infectivity decreased by 25%) and assume that biting rate is decreased by 25%, overall transmission is reduced by about 80%.

As the impacts on these traits differ among isolates, we could choose the isolate that is expected to lead to the greatest reduction of malaria transmission. In an ideal and simple world, this would be the isolate that affects each trait to the greatest possible degree. However, experiments suggest that the effects of the microsporidian are constrained by interactions among traits. Thus, for example, the isolates that reduce adult longevity most are those that are least likely to kill juveniles (Fig. 12.1D). Would the most effective control be achieved with an isolate that greatly reduces adult longevity, that reduces juvenile mortality or that has an intermediate effect on the two traits? According to the Ross-Macdonald equation (which suggests that transmission is most sensitive to adult mortality), isolates that greatly reduce adult longevity would be most effective at reducing malaria transmission. But this prediction depends on the shape of the interactions between the two traits and is influenced by possible interactions with the other epidemiologically relevant traits. A quantitative analysis of this question is lacking.

12.3. SUSTAINABLE CONTROL

That microsporidians affect mosquitoes in several ways that decrease their reproductive success leads to the concern that the evolution of resistance to the microsporidian is inevitable, decreasing the efficacy of control to the point where transmission could return to the pre-control intensity. However, this system offers several possibilities that may make the evolution of resistance less damaging or may indeed be beneficial for malaria control.

Let us, then, consider the evolutionary response of mosquitoes to the intensive use of a microsporidian parasite as an agent of control. First, it should be noted that resistance to infection by microsporidians, in particular against *V. culicis*, has not been observed in mosquitoes (Hansen and Koella, 2003; Michalakis *et al.*, 2008). Rather than being resistant to or

clearing an infection, mosquitoes can be tolerant of infection, that is, suffer less from its deleterious effects. As some mosquitoes are more tolerant than others, and this variation is partly determined genetically, it is almost inevitable that intensive use of microsporidians increases the level of tolerance. However, tolerance may be involved in trade-offs; tolerance at one life stage could constrain the tolerance at other life stages or influence other evolutionarily and epidemiologically important traits. This could lead to the counter-intuitive result that the evolution of tolerance has little, if any, impact on the success of control. As we lack evidence for such trade-offs, the ideas outlined below are hypothetical. Nevertheless, they show the power of using evolutionary concepts to influence ideas about disease control.

Consider, for example, the possibility that mosquitoes that are tolerant to microsporidians are also resistant to malaria. Such a link is suggested by the observation that microsporidians affect the nitric oxide (NO) synthase pathway (Biron *et al.*, 2005), which is involved in resistance to malaria (Luckhart *et al.*, 1998, 2003). We are in the process of investigating the association with experimental infections and with quantitative trait loci (QTL) mapping of field-caught mosquitoes. If tolerance to microsporidians and resistance to malaria share part of their genetic basis, the evolution and spread of tolerance to microsporidians would lead to mosquitoes that are more resistant to malaria and thus to less intense transmission.

To understand the interaction between the evolution of tolerance and malaria transmission more clearly, we simplify the situation by considering the mosquito's tolerance to two of the detrimental effects of microsporidian infection: higher juvenile mortality and shorter adult life span. If the mechanisms to achieve tolerance to these two effects are independent, evolution can (and will) lead to high tolerance to both. However, how will evolution proceed if tolerances of the juveniles and of the adults constrain each other, so that high tolerance in both is not possible? Will evolution then reduce the detrimental effect on juveniles or on adults? The answer is suggested by one of the best-accepted evolutionary concepts: selection pressure decreases as individuals age (Charlesworth, 1980, 1993). On the one hand, larval mosquitoes have all of their reproductive life ahead of them; they have no evolutionary fitness unless they are tolerant to microsporidian infection and survive at least up to having laid a first batch of eggs. On the other hand, once ageing adults have achieved most of their reproductive success, any increase in their longevity would enhance their evolutionary fitness only slightly. Therefore, there is much weaker selection pressure for mosquitoes to evolve tolerance as adults than as juveniles, so that the intensive use of a microsporidian as a control agent should lead to widespread tolerance of juveniles.

Changes in the adults' longevity, therefore, are unlikely to be the direct consequence of selection, but would follow the tolerance of juveniles via

the genetic constraints and trade-offs among life-history traits. While there are no data on the genetic correlation between juvenile tolerance and adult longevity, a combination of several independent datasets suggests that increased tolerance of juvenile mosquitoes may indeed constrain adults to have a shorter life span. First, microsporidian-infected larvae can increase the likelihood of surviving to emerge as adults, that is, can increase their tolerance, by shortening their larval period (Hansen and Koella, 2003), thereby giving the microsporidian less time to kill the mosquito before it emerges. The genetic association between tolerance and larval development is confirmed by artificial selection of *Aedes aegypti* for more rapid or slower development, which brings with it lower or higher juvenile mortality induced by the microsporidian *Edhazardia aedis* (Koella and Agnew, 1999) (Fig. 12.2A). Second, as is the case for many insects, age at pupation of mosquitoes is genetically correlated with the size of adults (Voordouw *et al.*, 2008) (Fig. 12.2B). Third, larger mosquitoes are expected to live longer than smaller ones (Ameneshewa and Service, 1996). Although we have found no data showing that such a relation has a genetic basis, we have shown a genetic correlation between adult size and the efficacy of the melanisation immune response (Voordouw *et al.*, 2008) (Fig. 12.2C). As individuals with a more effective immune response may be better at dealing with wounds and with parasites, the (genetically) larger mosquitoes may also live longer. It should be noted however, that like many insect immune responses (reviewed in Schmid-Hempel, 2005), the melanisation response of mosquitoes can be costly. Indeed, the melanisation response decreases fecundity in some cases (Schwartz and Koella, 2004), and may decrease the longevity of mosquitoes (Boëte *et al.*, 2004). Thus, despite the obvious benefits of immune responses, it is not clear that a stronger melanisation response would be directly associated with longer longevity. Nevertheless, even if the stronger melanisation response of larger mosquitoes is costly, it may indicate longer life span, for the ability of mounting a strong response may indicate general vigour. Clearly, these aspects require further study. If there indeed is a genetic path leading from tolerance through earlier pupation, smaller adults and less effective immune responses to adults with shorter life spans, a plausible evolutionary outcome of the use of microsporidians for malaria control is increased tolerance to microsporidians in juveniles associated with shorter adult life span.

How will this evolution affect the transmission of malaria? On the one hand, increased tolerance, in itself, increases the transmission of malaria, as it increases the number of adult mosquitoes; on the other hand, shorter-lived adults decrease the transmission of malaria. To evaluate the overall epidemiological consequences of the evolution of tolerance, we use the equation underlying transmission: $R_0 = \frac{ma^2be^{-\mu T}}{r\mu}$. As mentioned above,

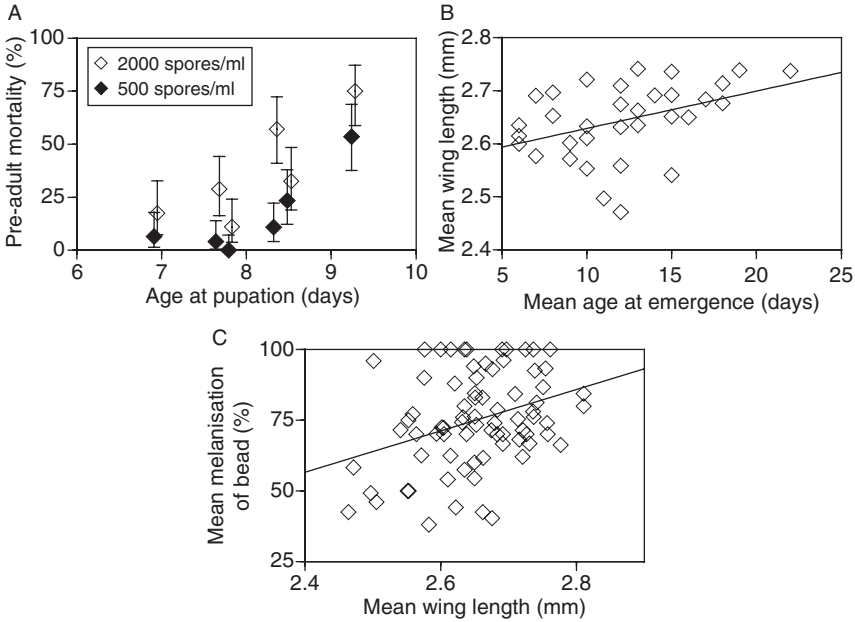

FIGURE 12.2 Series of data suggesting that juvenile tolerance to microsporidians is genetically correlated to adult longevity. (A) The juvenile mortality induced by the microsporidian *Edhazardia aedis* in lines of *Aedes aegypti* selected for earlier or later pupation (and thus differing genetically) (generated from data in Koella and Agnew, 1999). (B) The mean wing length of full-sib families of *Anopheles gambiae* as a function of their mean age at emergence (generated from data in Voordouw et al., 2008). As families differ genetically, the correlation has a genetic basis. (C) The efficacy of the melanisation response (percentage of a Sephadex bead covered by melanin) of full-sib families of *Anopheles gambiae* as a function of their mean wing length (generated from data in Voordouw et al., 2008). As the melanisation response is essential for wound healing and deals with infections, more effective melanisation is likely to be associated with a longer life span.

reducing the longevity of adults is more efficient at controlling malaria than killing larvae to reduce the number of mosquitoes. Therefore, an increased number of adult mosquitoes associated with tolerance can be offset by a slight decrease in longevity. This is shown in Fig. 12.3, which illustrates whether changing mosquito density and adult longevity by the factors given on the axes increases (white area of Fig. 12.3) or decreases (grey area of Fig. 12.3) the intensity of transmission.

Although we do not yet have the data to support this idea, we may speculate that malaria control with microsporidians may indeed be evolution-proof in the sense that evolution will not lead to a failure of control. Although the evolution of tolerance by the mosquitoes would

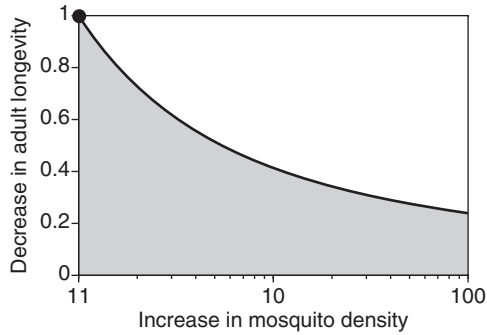

FIGURE 12.3 The effect on the intensity of transmission R0 of reducing adult longevity or increasing mosquito density (i.e., reducing juvenile mortality). The circle gives the initial parameters (no change). Increasing mosquito density or adult longevity enhances transmission. Increasing mosquito density while decreasing adult longevity to the value given by the curve has no affect on transmission. A combination of changes above the curve enhances transmission; a combination below the curve (in the grey area) reduces transmission. Thus, greatly increased mosquito density (tolerance to the microsporidian) can be compensated by a moderate reduction of adult longevity.

imply that the microsporidians have less direct effect on the transmission of malaria, the indirect effects via trade-offs in life-history parameters would ensure that the intensity of transmission remains low.

12.4. CONCLUSIONS

While we speculate about the possible use of microsporidians for the control of malaria, our aim is more general. First, we emphasise that effective control is possible with a control agent that targets several epidemiologically relevant parameters. This conclusion is not surprising, and indeed many modern control programmes use integrated approaches with multiple tools for malaria control. Second, evolution need not lead to the failure of control. The two critical components that let evolution be helpful for control are (i) that the epidemiologically relevant aspect of control (here, adult longevity) differs from the evolutionary target (here, juvenile tolerance) and (ii) that the two traits are negatively genetically correlated.

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