Ecology of parasite-vector interactions

edited by:

Willem Takken and Constantianus J.M. Koenraadt

Ecology and control of vector-borne diseases
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Ecology and control of vector-borne diseases

In the past century, many advances were made in the control of vector-borne diseases. Malaria disappeared from the northern hemisphere, diseases such as typhus, *Bartonella* and yellow fever were seriously reduced in prevalence and in many countries effective methods of disease control contributed to a greatly reduced incidence of such diseases. Most of these advances were beneficial to the industrialised world, whereas underdeveloped countries continued to suffer much as before. Indeed, several diseases such as malaria, Rift Valley fever and African sleeping sickness are still highly prevalent in parts of the tropics. 'New' vector-borne diseases such as dengue, chikungunya fever and West Nile fever, have emerged and are invading previously disease-free regions. The discovery of new drugs and vaccines has made great advances and allows for the effective treatment and control of many diseases. In contrast, vector control has lagged behind in development, even though it is realised that effective vector control would allow for an immediate interruption of the transmission of disease, and aid in disease control and eradication. In the last decade new initiatives on vector control have been undertaken, leading to a rapid development of effective and lasting methods of vector control. For example, the Roll Back Malaria control programme of the World Health Organization has led to significant reductions in malaria in many countries. In order to achieve further advances, however, additional tools are required. The development of molecular genetics has provided new insight in vector biology and behaviour, which is being used for developing new strategies of vector control. Advances in geographic information systems allow for precision targeting of interventions. The collective information on new developments in Vector Ecology and Control for Vector-borne Diseases is scattered over numerous periodicals and electronic databases. This book series intends to bring together this information in sequential volumes arranged around selected themes that are currently of interest. Forthcoming themes will include 'Recent advances in biological control of mosquitoes', 'Transgenic tools for vector management' and 'Integrated management of vectors of livestock diseases', but also fundamental biological topics such as 'Mating behaviour of disease vectors', 'Oviposition behaviour of disease vectors' and 'Reproductive strategies of disease vectors'. Other topics will be added as perceived relevant.

Willem Takken is the senior editor of the series. Each volume will be co-edited by a guest editor, which in Volume 3 is Sander Koenraadt. The editors of the current volume are well-known experts in the field of Medical and Veterinary Entomology, and have experience from field work in the tropics and ecological studies in laboratory and field. Willem Takken is professor in Medical and Veterinary Entomology at Wageningen University. Sander Koenraadt is an assistant professor in Vector Ecology at Wageningen University. Both editors collaborate in several research programmes, and consider dissemination of research results to fellow scientists as well as the public at large as an important component of their work.

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Preface

The current book is the 3rd volume of the series Ecology and Control of Vector-borne Diseases. The series is intended to cover a wide series of topics that concern vector-borne diseases, from fundamental research to control. By focusing on specific subjects in each Volume, an in-depth and up to date information about these subjects is provided, which can help to gain a perspective of recent advances in knowledge and how this can be exploited for effective disease control.

When the series was launched in 2007 (ECVD Vol. 1), we could not imagine that vector-borne diseases would dominate the international scene at a scale as we have witnessed in the last few years. Following the 2006 outbreak of bluetongue virus in north-western Europe, outbreaks of other vector-borne diseases in Europe followed each other with increasing speed: chikungunya in Italy (2007) and then in France (2010), dengue in France (2010) and recently in Greece (2012) for the first time since the nineteen twenties, new strains of West Nile virus in southern Europe (2011, 2012) and Usutu virus advances in Germany (2012). In the USA, Florida has experienced serious outbreaks of dengue in 2010, while Texas recently experienced its worst epidemic of West Nile virus since the disease arrived in the USA in 1999. In the tropics, where spectacular advances have been made in the control of malaria, insecticide and drug resistance threaten to halt this process. Of equal concern is the global advance of dengue, which has become a disease of the urban environment. Not only do vector-borne diseases emerge and expand their geographical territories, also some of the vector species migrate across huge distances. *Aedes albopictus* is probably the best example of a highly successful global migrant, and is now likely to become established in Australia as well due to its highly competitive nature. In Europe, *Aedes japonicus* has become established in Switzerland, and small pockets of this species were found elsewhere on the continent as well.

The realisation that vector-borne diseases continue to be prevalent and are capable of rapidly invading novel geographic areas, is reason to continue investing in research on the basic biology of these diseases as well as in the development of preventive and effective control measures. Variation in parasite and vector genetics and ecological determinants provide challenges that need to be met. The current volume of ECVD focuses on the interaction between parasites and vectors. As several of the chapters will show, rapid advances in molecular genetics allow for some revision of our classical knowledge about vector-parasite interactions and provide insight in the intricate regulation of these interactions, notably the role of immune responses as well as endosymbionts. Better insight in these processes can provide the key to successful interruption of these interactions and hence to disease control.

Many scientists have contributed to this Volume, and we appreciate the time taken to complete these chapters, which show strong interlinkages about the subject of parasite-vector interactions. Each chapter was reviewed by independent reviewers, and subsequently adjusted as needed. We are grateful to the anonymous reviewers, as we realise that this takes time and effort. We thank Wageningen Academic Publishers for their patience and useful advice. We thank Hans Smid for once more having contributed to the cover design.

Wageningen, 22 October 2012

Willem Takken and Constantianus JM (Sander) Koenraadt

1. Introduction – who was there first?

Willem Takken and Constantianus J.M. Koenraadt

Abstract

The force of vector-borne disease transmission is greatly affected by interactive processes between parasites and their arthropod hosts. In recent years significant advances in knowledge about the mechanisms of these interactions have been made, notably concerning the impact of arthropod immune responses on parasite establishment and propagation in the arthropod host, genetic and phenotypic variation affecting these interactions, the impact of these interactions on parasite and arthropod fitness, and how environmental factors affect parasite transmission. The current volume of the Ecology and Control of Vector-Borne Diseases highlights significant and novel aspects of parasite-vector interactions and contributes to a better understanding of vector-borne disease transmission. Better insight in these interactive processes will be useful for studies on the epidemiology and control of vector-borne diseases and is expected to contribute to the development of novel intervention strategies.

Keywords: vector-borne disease, parasites, pathogens, interaction, genetics, immunity, transmission

Introduction

Vector-borne diseases are characterized by the fact that one organism is dependent for its existence on at least two other organisms, one vertebrate host and one arthropod host. Examples are the leishmaniases with rodents as the vertebrate reservoir and sandflies (Diptera: Phlebotominae) as the arthropod hosts, dengue with humans as the vertebrate reservoir and *Aedes* mosquitoes (Diptera: Culicidae) as arthropod hosts and African trypanosomiasis, with various mammalian species as reservoir hosts and tsetse flies (Diptera: Glossinidae) as arthropod hosts. Historically, much attention has been paid to the parasite-vertebrate host interaction, as knowledge about this association is essential for understanding of the disease and contains clues for treatment. This includes research on the biology of parasites, the onset and progress of clinical disease, and preventive and curative interventions. Conversely, the parasite-arthropod interaction has received far less attention, presumably because it was originally assumed that passage of the parasite through the arthropod would not affect the latter. With hindsight, this was a remarkable attitude, as it was realized soon after the discovery of the role of arthropods as vectors that the parasites would require nutrients from their hosts for survival and reproduction (reviewed by Hurd 1990).

The realization that parasites can manipulate their arthropod hosts, or that the arthropods elicit effective immune responses against invading parasites coupled with rapidly-advancing technologies for parasite detection and identification (Sim *et al.* 2009, Valkiunas *et al.* 2008), has led to a growing body of research on parasite-vector interactions that has assisted greatly in our understanding of the biology of vector-borne diseases. For example, the development of real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) techniques has led to the discovery of sub-microscopic infectious *Plasmodium* stages in the human host, suggesting that malaria parasite transmission may occur at a much greater scale than was believed hitherto (Schneider *et al.* 2007). Advances in immunology allow for a detailed understanding of the infectious route of *Plasmodium* spp. in anopheline mosquitoes (Chapter 2, Cirimotich *et al.* 2010). Molecular tools revealed the highly complex interactions between trypanosome parasites and their tsetse fly hosts, showing the effect of symbiotic interactions on successful parasite establishment following

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an infectious blood meal, as well as parasite multiplication and passage to the salivary gland (Aksoy and Rio 2005). These advances affect not only the true parasite-vector interactions, but also those involving true pathogens such as viruses, bacteria and fungi. Recent studies demonstrated the role of cellular mechanisms of *Ixodes ricinus* (L.) ticks (Acari: Ixodidae) in the establishment and subsequent multiplication of *Borrelia burgdorferi* spp. in the tick host (Schuijt *et al.* 2011), or of the impact of the mosquito host on the replication of dengue virus (Sim and Dimopoulos 2010). New insights about parasite-host interactions are not limited to direct interactions at the dual level of parasite and arthropod host, but also about environmental factors such as temperature, humidity and symbionts. For example, it was recently shown that daily temperature fluctuations greatly affect the transmission of *Plasmodium* (Chapter 5, Paaijmans *et al.* 2012) as well as dengue virus (Lambrechts *et al.* 2011) by their respective mosquito vectors. The relevant role of endosymbionts in the regulation of parasite-vector interactions is becoming increasingly realized, especially as the symbionts not only provide essential nutrients to the arthropod host, but also affect immune responses as well as the fitness of the parasite (Chapter 2, Hughes *et al.* 2011, McMeniman *et al.* 2009, Pinto *et al.* 2012). Recently, it was discovered that *Chromobacterium* spp. present in the midgut of mosquitoes possibly release anti-pathogenic agents that kill *Plasmodium* parasites as well as dengue virus (G. Dimopoulos, personal communication) and it is likely that microbial-vector interactions affect the successful establishment of parasites in the vector in ways that are not yet understood.

These examples are only a fraction of the vast amount of knowledge on parasite-vector interactions that has emerged in recent years. Such knowledge is likely to affect our understanding of vector-borne disease epidemiology, and can potentially be used for more effective interventions aimed at the control of vector-borne diseases. The revelation of malaria hot spots (Chapter 11, Bousema *et al.* 2012)) was only made possible through these advancements and provides new opportunities for more effective, targeted malaria control. The insertion of *Wolbachia pipientis* Hertig in *Aedes aegypti* (L.) has revealed a novel implementation of endosymbionts for the control of dengue virus (Frentiu *et al.* 2010) and will shortly be tested in dengue-endemic areas (S. O'Neill, personal communication).

The chapters in this third volume of ECVD highlight current advances in research on parasite-vector interactions, and provide proof that such advances are essential for the improvement of current disease control strategies. With the rapid advancement of insecticide resistance against malaria vectors (Asidi *et al.* 2012, Ranson *et al.* 2011) coupled with increasing drug resistance and the apparent spread of vector-borne diseases associated with environmental change (see Takken and Knols 2007), effective tools for vector-borne disease control are urgently needed, and studies that lead to better understanding of parasite-vector interactions will contribute to achieve that goal.

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Fundamental aspects of vector-parasite interactions

2. Impact of transgenic immune deployment on mosquito fitness

Andrew D. Pike, Chris M. Cirimotich and George Dimopoulos

Abstract

Mosquitoes are the vectors of pathogens causing numerous human diseases, including dengue and malaria. Due to increases in drug resistance among pathogens or the lack of effective treatments for these diseases and increasing insecticide resistance among mosquito populations, new methods of control are urgently needed to limit the morbidity and mortality caused by vector-borne diseases. Mosquitoes possess an innate immune system capable of limiting infection with human pathogens, and the creation and deployment of transgenic mosquitoes with an enhanced immune system has been suggested as a novel means to reduce mosquito vector competence. However, activation of the immune system is often associated with a cost to the host, which could limit the ability of the transgenic insects to replace their wild-type conspecifics. Here, we discuss recent research into the effects of increased immune deployment and insect transgenesis on the fitness of the mosquitoes.

Keywords: insect transgenesis, fitness, innate immunity, vector-borne disease, vectorial capacity, *Drosophila*, *Anopheles*

Introduction

Mosquitoes serve as vectors for numerous viral, filarial and protozoan pathogens of great public health importance in both humans and other animals. Principal among these agents are the *Plasmodium* parasites responsible for malaria and the viruses responsible for dengue fever and dengue haemorrhagic fever/dengue shock syndrome. Mosquitoes become infected with these pathogens when acquiring a blood meal from an infected vertebrate host and, following an incubation period during which the pathogen develops or replicates within the vector mosquito, they transmit the pathogens to a susceptible host during a subsequent blood meal.

Many factors contribute to the ability of a mosquito to successfully transmit a pathogen and to the efficiency of disease transmission, referred to as the vectorial capacity. The inherent capability of a mosquito to transmit the pathogen, or vector competence, is determined by genetic components of the mosquito and pathogen as well as by environmental components, including temperature. Other variables involved in vectorial capacity include the vector population density, the extrinsic incubation period required for vectors to become infectious and the daily survival rate of competent vectors. A mathematical model of vectorial capacity was first described by Ross (1911) and later refined by MacDonald (1957) and Smith and MacKenzie (2004). The full equation is given by

$$C = \frac{\beta m a^2 p^n}{-\ln p},$$

where C is the vectorial capacity, β is the vector competence, m is the density of the vector population, a is the daily biting rate, n is the extrinsic incubation period of the pathogen and p is the daily survival rate. A change in any one of these variables can greatly affect the overall vectorial capacity for a specific pathogen and alter the persistence of the disease.

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The advent of molecular biology, genomics and functional genomics has provided unprecedented opportunities to elucidate the complex interactions that take place between the mosquito vector and the pathogens it transmits. These technologies have led to significant advances in our understanding of how the mosquito's innate immune system is actively involved in killing large fractions of these human pathogens, sometimes rendering the mosquito vector completely refractory to infection. The progress made in basic research, together with the development of mosquito transgenic methodologies, has opened the way for the development of novel disease control strategies that are based on blocking pathogen transmission in genetically modified immune-enhanced mosquitoes. However, despite the ongoing development of powerful genetic drive systems, a potential bottleneck in the course of successful deployment of genetically modified pathogen-immune mosquitoes is the possible impact of the immune transgene on mosquito fitness. This chapter will address our current understanding of the interactions between the insect immune system and the organism's fitness and how that interplay might influence the use of genetically modified pathogen-immune mosquitoes for vector-borne disease control.

The mosquito innate immune system

Infection of a mosquito with a parasite or virus has a profound effect on the transcriptional repertoire of the mosquito. Hundreds of genes are regulated and implicated during infection, especially those encoding factors involved in the mosquito innate immune response (Dong *et al.* 2006, Xi *et al.* 2008). Mosquito genetics play a crucial role in vector competence, and especially in the inherent ability of the mosquito to mount an effective neutralizing immune response against the invading pathogen. Unlike humans and other mammals, mosquitoes do not have genes for the production of antibodies and other molecules of the adaptive immune response. Instead, the mosquito's innate immune system directly responds to and combats pathogens upon challenge. Pattern recognition receptors (PRRs) on the surface of immune-competent cells, or circulating in the haemolymph, bind to specific pathogen-associated molecular patterns (PAMPs), triggering a series of reactions that culminate in the expression of anti-pathogen effector molecules. The ultimate result of immune pathway activation is an up-regulation of specific gene expression that is PAMP- and pathway-dependent. These immune effector genes form an important line of defence for the mosquito against a variety of invading pathogens. PRRs can also directly activate immune defence mechanisms such as phagocytosis and complement-like killing mechanisms, independent of the intracellular immune signalling pathways.

Various cellular and humoral factors in the mosquito haemolymph play a significant role in the response to microbial challenge. Circulating immune-competent cells, known as haemocytes, phagocytose and encapsulate foreign particles and pathogens. Simultaneously, serine protease cascades activate enzymes that generate melanin and free radicals, which are responsible for killing microbes during humoral responses. These effectors create a series of barriers that a pathogen must surmount before the mosquito becomes infectious, and an increase in any of these anti-pathogen factors can greatly reduce the vector competence of the mosquito.

A number of immune signalling pathways regulate anti-pathogen immunity in mosquitoes. With the advent of whole-genome sequencing projects over the past decade (Holt *et al.* 2002, Nene *et al.* 2007), the three major known immune signalling pathways (Toll, IMD, and Jak/Stat) that were originally described in *Drosophila* or mammals have been identified through orthology in mosquitoes (Christophides *et al.* 2001).

The Toll pathway has been implicated in the mosquito defence against fungal, bacterial, parasitic and viral infections (Antonova *et al.* 2009, Shin *et al.* 2005, Xi *et al.* 2008). PAMP recognition by Toll pathway PRRs is well documented, but the underlying mechanism is still unresolved. The *Drosophila* genome encodes two distinct Toll pathway-regulated transcription factors, *Dif* and *Dorsal*, which mediate immune and developmental gene expression, respectively. The *Aedes aegypti* (L.) genome also encodes two distinct Toll pathway transcription factors (REL1A and REL1B), while the *Anopheles gambiae* Giles genome encodes a single factor (REL1/GAMBIF1) (Barillas-Mury *et al.* 1996, Shin *et al.* 2002).

It has been shown through transient activation of the Toll pathway via silencing of the negative regulator *cactus* that Rel1-transcribed effector molecules are critical for the *Ae. aegypti* defence against dengue viruses (Ramirez and Dimopoulos 2010, Xi *et al.* 2008) and the *An. gambiae* defence against rodent malaria parasites (Frolet *et al.* 2006, Garver *et al.* 2009, Meister *et al.* 2005, Zou *et al.* 2008). Frolet *et al.* (2006) and Garver *et al.* (2009) used this transient immune stimulation to show that Toll pathway activation decreases the *Plasmodium berghei* parasite burden, whereas depletion of the Rel1 transcription factor increases infection levels in mosquito midguts. Frolet *et al.* (2006) suggest that Toll pathway-regulated effector molecules are constantly in circulation and can immediately attack an invading pathogen. Transcriptional activation subsequent to pathogen challenge is then used to replenish molecules used during the initial insult (Frolet *et al.* 2006). However, Toll pathway-mediated killing of parasites may not be relevant to all parasite species. *P. berghei* infection of *An. gambiae*, *Anopheles stephensi* Liston and *Anopheles albimanus* Wiedemman, as well as *Plasmodium gallinaceum* Brumpt infection of *Ae. aegypti* appear to be controlled through Toll pathway activation, while *Plasmodium falciparum* infection of various anopheline mosquitoes is affected to a lesser degree by the Toll pathway (Garver *et al.* 2009, Zou *et al.* 2008).

Initiation of signalling through a second innate immune pathway, the immune deficiency (IMD) pathway, protects mosquitoes from infection with *Plasmodium*, especially the human malaria parasite *P. falciparum* (Dong *et al.* 2011, Garver *et al.* 2009, Meister *et al.* 2005, 2009, Mitri *et al.* 2009). Signalling events within this pathway culminate in the expression of various effector genes mediated by the Rel2 transcription factor. Basal levels of IMD pathway-mediated gene expression are constantly regulated by a shortened splice variant of Rel2, while the full-length isoform is continuously present in the cell cytoplasm, but inactive until immune stimulation occurs (Luna *et al.* 2006, Meister *et al.* 2005). Pathway activation stimulates the cleavage of the full-length isoform, exposing the nuclear localization signal and causing nuclear translocation of the transcription factor and a subsequent increase in the transcription of immune effectors. Garver *et al.* (2009) used transient depletion of the negative regulator *caspar*, and Dong *et al.* (2011) used transgenic over expression of Rel2, to show that induction of the IMD pathway renders mosquitoes nearly refractory to *P. falciparum* infection. Interestingly, both the Toll and IMD pathways are mosquito species-independent, in that multiple mosquito species use the same pathways to combat pathogens, but are *Plasmodium* species-dependent.

The third major immune pathway, the Jak/Stat pathway, is named for the kinases (Jak) and transcription factors (STAT) that control its activation. The pathway has antiviral activity in *Ae. aegypti* (Souza-Neto *et al.* 2009) and can control *Anopheles-Plasmodium* interactions during the later stages of infection (Gupta *et al.* 2009). Interestingly, in contrast to *An. gambiae*, the *Ae. aegypti* Jak/Stat pathway also controls *P. gallinaceum* infection at the early pre-oocyst stages (Zou *et al.* 2011). Two STAT transcription factors, STAT-A and STAT-B, have been identified in *An. gambiae*, while only one STAT is present in *Ae. aegypti*. In *An. gambiae*, STAT-B modulates the transcription of STAT-A, the ancestral transcription factor and predominant form in adult mosquitoes. Translocation

of STAT-A to the nucleus leads to up-regulation of anti-pathogen effector molecule expression. In experiments similar to those described above for the Toll and IMD pathways, activation of the Jak/STAT pathway via depletion of the negative regulator *SOCS* decreases the density of *P. berghei* late oocysts, indicating that the pathway is important for anti-*Plasmodium* responses in *Anopheles* malaria vectors (Gupta *et al.* 2009).

Activation of any innate immune pathway leads to an increase in the production of various anti-pathogen molecules. A large number of anti-*Plasmodium* effector molecules have been identified, including leucine-rich repeat domain-containing proteins, fibrinogen-related proteins, C-type lectins, and others (reviewed in Cirimotich *et al.* 2010). Of particular interest is the thioester-containing protein TEP1, which has been shown to be crucial for mosquito defence against *Plasmodium* parasites (Levashina *et al.* 2001). TEP1, a homolog to the vertebrate complement system molecule C3, is constitutively secreted by haemocytes into the mosquito haemolymph, allowing it to interact with pathogens soon after they infect the mosquito (Levashina *et al.* 2001). Once a pathogen is detected, TEP1 binds to the surface of the invading microbe and promotes phagocytosis and, therefore, clearance of the intruder (Moita *et al.* 2005). TEP1 expression is induced in response to both Toll and IMD pathway activation, reflecting the molecule's importance in mosquito innate immune responses (Blandin *et al.* 2004, Garver *et al.* 2009, Levashina *et al.* 2001).

The dissection of the mosquito immune response to human pathogens has led to the discovery of immune pathway factors and downstream anti-pathogen effectors that can potentially be used to render the mosquito resistant to these infections through transgenic tissue- and infection stage-specific over-expression.

Mosquito transgenesis

The introduction of novel genetic elements into mosquito genomes has become a powerful approach for the study of mosquito immunity and has the potential to be used for future control of mosquito populations and to reduce the vectorial capacity of mosquitoes for human pathogens. In transgenesis, a mobile DNA element is used to introduce a gene of interest into the mosquito germline. This gene of interest is placed under the control of a specific promoter, which determines the tissue specificity and temporal expression of the transgene making it possible to express the gene with temporal and spatial specificity, or only when induced and only in certain tissues. Tools and methodologies for the genetic modification of *Anopheles* and *Aedes* mosquitoes have been developed and widely used to study various aspects of the vectors' biology. Successful transformation of mosquitoes was first achieved in *Ae. aegypti* (Coates *et al.* 1998, Jasinskiene *et al.* 1998) and soon followed by *An. stephensi* (Catteruccia *et al.* 2000), *An. gambiae* (Grossman *et al.* 2001) and *An. albimanus* (Perera *et al.* 2002), leading to the creation of many different strains of transgenic mosquitoes in each of these species.

In *Aedes* mosquitoes, transgenesis has been successfully used to identify Rel1-driven gene expression as a major component of anti-fungal immunity (Bian *et al.* 2005). It has also been used to show that Rel2-driven gene expression provides a defense against systemic bacterial challenge and *P. gallinaceum* infection (Antonova *et al.* 2009, Shin *et al.* 2003) and that RNA interference is crucial for antiviral defence (Khoo *et al.* 2010). Transgenesis has been utilized to study the innate immune pathways of *Anopheles* mosquitoes as well, and to express heterologous genes for the purpose of altering vector competence in both *Aedes* and *Anopheles* mosquitoes (reviewed in Cirimotich *et al.* 2011, Dong *et al.* 2011).

In order to affect the mosquito's vectorial capacity for a given pathogen, transgene expression must be driven in a relevant tissue, for instance the midgut, and at the appropriate time, i.e. when the pathogen has invaded that particular tissue. Midgut-, fat body- and salivary gland-specific promoters have been utilized to decrease vector competence in transgenic mosquitoes (Dong *et al.* 2011, Franz *et al.* 2006, Isaacs *et al.* 2011, Mathur *et al.* 2010). Mating of separate transgenic lines may eventually be used as a strategy to induce transgene expression in a single mosquito at multiple time points and locations, increasing the chances that pathogen development will be negatively affected and minimizing the possibility that the pathogens will be able to evade the immune response.

The implementation of transgenic technologies that utilize the mosquito innate immune system to combat vector-borne disease can largely be achieved in three ways: (1) over-expression of a pathway activator, such as a NF-κB transcription factor, to turn on the expression of anti-pathogen molecules; (2) depletion of negative regulators of a pathway through the expression of a hairpin transgene, again activating that specific pathway; and (3) over-expression of immune genes/effector molecules that directly affect the pathogen. Each approach has advantages and disadvantages, but regardless of the mechanism, the end result is a less suitable host environment for pathogen development.

The first and third strategies have previously been used experimentally in both *Ae. aegypti* and *An. stephensi* to demonstrate that this principle may eventually be applied to the engineering of pathogen-resistant mosquito populations (Antonova *et al.* 2009, Dong *et al.* 2011, Kokoza *et al.* 2010). As mentioned above, Rel2 has been over-expressed in *Ae. aegypti* in order to impede the development of *P. gallinaceum* parasites (Antonova et al, 2009). When Rel2 is expressed under the control of the vitellogenin promoter, which is inducible in the fat body of the mosquito upon blood-feeding, the transcription of a number of antimicrobial peptides (AMPs) is induced. These transgenic mosquitoes are more resistant than non-engineered mosquitoes to the establishment of *P. gallinaceum* infection in the midgut and sporozoite production in the haemolymph (Antonova *et al.* 2009). In follow-up studies, Kokoza *et al.* (2010) engineered *Ae. aegypti* mosquitoes to over-express the AMP genes *cecropin A* and *defensin A* directly, rather than inducing the entire Rel2-mediated pathway. Separate transgenic mosquito lines were engineered to induce either AMP gene singly or both together under the control of the vitellogenin promoter. Regardless of the configuration, transgenic expression of these genes decreased parasite development and completely abolished the vectorial capacity of the mosquitoes for parasite transmission, as measured by sporozoite production (Kokoza *et al.* 2010). Similar strategies have also been pursued in *An. stephensi* mosquitoes. Dong *et al.* (2011) created *An. stephensi* that overexpress Rel2 under the control of both the carboxypeptidase and vitellogenin promoters, leading to an increase in the ability of the mosquitoes to fight off both *Plasmodium* and bacterial infections.

Although candidate molecules exist for the development of mosquito refractoriness to various pathogens, the eventual release of genetically modified mosquitoes into the environment must coincide with the development of effective drive mechanisms to force the spread of the transgene into the native mosquito populations. The drive mechanism must have the power to drive the transgene to near-fixation in the native population and be sufficiently well-linked to the transgene to avoid separation from it. The current list of potential drive mechanisms includes transposable elements, homing endonuclease genes and the *Medea* system (reviewed in Cirimotich *et al.* 2011). The ability of a transgene to spread within mosquito populations will also be significantly affected by any fitness cost associated with transgene integration and expression.

Impact of insect immune system activation on its fitness

Although the immune system is vital to fighting off infection by various invading pathogens, long-term or constitutive over-expression of immune genes can negatively affect the host. This situation has been best described in autoimmune diseases of mammals, but it also occurs with the innate immune system of insects (DeVeale *et al.* 2004). Similarly, transgenic expression of heterologous genes or over-expression of endogenous genes can engender various fitness costs if the endogenous gene expression is interrupted or maintenance of transgene expression requires the use of necessary resources (Marrelli *et al.* 2006). In order to utilize transgenesis as a control mechanism for vector-borne disease in mosquitoes, the fitness effects of both the innate immune response to pathogens and the specific transgene expression must be assessed and understood. This will allow the creation of transgenic refractory vectors that can successfully compete with wild-type conspecifics and invade the natural population.

Fitness, most commonly indicated by the net reproductive rate, is considered to be the sum of many complex interactions that make it possible for an organism to reproduce. The two main components of fitness are lifespan and fecundity, or the ability to produce offspring successfully (Marrelli *et al.* 2006). Lifespan, or the length of time a disease vector is able to survive in its environment, is important because it determines the number of times an individual will be able to reproduce, whether the pathogen will have sufficient time to develop into an infectious stage and, finally, whether that individual is able to feed multiple times on blood in order to transmit the pathogen from one host to another. Daily survival is a particularly important component of vectorial capacity, since a vector that dies before the end of the pathogen's incubation period will be unable to transmit the pathogen. The significance of survival time is reflected in the fact that the daily survival rate is an exponential term in the vectorial capacity equation, indicating that even small changes in survival can lead to large changes in vectorial capacity for any pathogen transmitted by the mosquito (MacDonald 1957). Fecundity, usually measured by the number of viable offspring produced by an individual, is determined by both the number of times an organism is able to reproduce and the number of offspring that are produced during each reproductive cycle. If the fecundity and lifespan of a mosquito species decrease, the vectorial capacity will also be reduced, since the density of the vector will be reduced, and so will the chance that the mosquito will bite people. Both of these parameters are, in turn, affected by a large number of other factors, including the mosquito's ability to avoid predators, combat disease and allocate energetic resources.

Given the limited nutritional resources available to an organism during its life, an increase in the use of energy reserves for one purpose must lead to the reallocation of those resources from another area of activity. For instance, if an insect starts over-expressing certain immune effector genes in response to infection, or as a result of transgenesis, the raw materials and energy used to make those immune-related proteins must come from some other area. Similarly, if the insect has *X* arbitrary units of energy to use to produce all the proteins it needs to survive and reproduce, creating new proteins will utilize more of this energy. If the induction of the immune system causes more than *X* energy units to be used, the energy must be taken from that previously used to produce other proteins. This reallocation of resources could lead to a decrease in the ability of the insect to reproduce or a reduction in its lifespan, rendering it 'less fit' than an insect without immune activation. Infection of *Drosophila* as well as mosquitoes has been shown to alter the expression of hundreds of genes with diverse functions, indicating that the effects of infection are wide-ranging and not limited to immune deployment (Aguilar *et al.* 2005, De Gregorio *et al.* 2001, Dong *et al.* 2009). Because the factors that define fitness are only loosely defined, and because

the widespread effects of immune deployment have not yet been fully described, the effects of an increase in insect immune gene expression on fitness are difficult to predict.

Numerous studies have measured the effects of *Drosophila* immune activation on fly longevity. Many of these studies have been conducted by injecting wild-type and transgenic flies with various types of bacteria and measuring the fecundity or lifespan of the fly after infection. A negative effect of immune induction on fitness has generally been observed, suggesting a trade-off between fitness traits and the ability to deploy an effective immune response. For instance, wild-type flies infected with *Escherichia coli* Castellani and Chalmers or *Micrococcus luteus* (Schroeter) Cohn at various ages laid fewer eggs than their uninfected conspecifics, and interruption of the immune response by mutation of the genes *relish* and *imd-1*, components of the Toll and IMD pathways, respectively, alleviated these costs (Zerofsky *et al.* 2005). These findings show that infection, together with the activation of the immune system, can decrease a female fly's ability to reproduce. Induction of the Toll pathway also leads to a reallocation of resources in the fat body, suggesting a mechanism for this fitness cost and supporting the trade-off between immunity and reproduction (DiAngelo *et al.* 2009). These negative effects are significantly increased in food-limited environments, again adding support to the hypothesis of an energetic exchange between immune response and fitness. When flies were infected with the Gram-negative bacterium *Providencia rettgeri* Brenner, those provided with unlimited food exhibited no negative fitness effects of infection, while those with a limited food supply showed an inverse correlation between resistance and fecundity (McKean *et al.* 2008). Similarly, infection of wild-type flies with *Serratia marcescens* Bizio resulted in significantly reduced lifespans in both male and female flies, but only female flies experienced a decrease in fecundity, despite equivalent bacterial loads (Imroze and Prasad 2011). While the increase in mortality was the same in both sexes, the lack of an effect on male fecundity implies that the cost of infection and immune deployment depend on other energy expenditures. Because male flies require comparatively less energy to mate with females, there may be less of an effect on their fecundity than in females, which must invest a greater amount of energy into production of eggs. However, males must also spend a significant amount of energy on attracting a mate and may experience more mating competition, and several studies have observed that infection has a greater effect on the lifespan of males than females, and that the extent of this effect is dependent on the relative availability of food resources (Bedhomme *et al.* 2004, Sharmila Bharathi *et al.* 2007).

Another study has shown that an increase in the sexual activity of a male fly is correlated with a decrease in the ability to fight off infection, again indicating a fitness cost of the immune activation (McKean and Nunney 2001). Males housed with a greater number of virgin females were less able to clear *E. coli* infections than were males exposed to fewer females. The authors suggest that this decrease in the ability to fight off an infection is a result of the flies' spending energy on mating instead of the immune response, consistent with the hypothesis that there is a trade-off between immune activation and fly fitness (McKean and Nunney 2001). This effect is not limited to bacterial infections. Fly larvae that successfully survive infection with the parasitoid wasp *Acyrthosiphon pisum* Harris exhibit a reduction size and fecundity as adults, as well as an increased susceptibility to parasitoids during the pupal stage (Kraaijeveld *et al.* 2002). Infection of flies with various pathogens has therefore been observed to lead to a decrease in the adult flies' lifespan and ability to reproduce, and the authors cite immune activation as the reason for these fitness costs (Kraaijeveld *et al.* 2002). If these observed costs in fitness are exclusively the result of immune activation, and not other influences that the pathogen may exert on the fly, the use of immune activation to limit insect infection will be difficult or impossible, since these high fitness costs would preclude refractory flies' invasion and maintenance in nature.

However, these studies largely depend on (1) infection of the fly with various pathogens to initiate an immune response and (2) an attribution of any observed fitness effects to the immune deployment alone, ignoring the fact that the infectious agent may have a significant impact on biological processes of the host other than immune activity. In fact, the injection of high numbers of bacteria directly into the haemolymph of a fly, and any subsequent replication of these bacteria, is certain to have effects on the fly that far exceed simple immune induction. The bacteria will, for example, utilize fly resources for their own replication and may produce virulence factors that directly alter fly fitness, irrespective of immune activation. Also, this model of immune deployment leads to long-term, if not lifelong, immune activation, while transgenic insects can be designed to have only transient activation of an immune defence mechanism in a specific tissue compartment. To this end, another study investigated the effects of short-term immune activation mediated by the NF-κB transcription factor, controlled by the IMD pathway, using an inducible system in transgenic flies (Libert *et al.* 2006). In this system, long-term immune activation by constitutive over-expression of peptidoglycan recognition protein (PGRP-LE) in the fat body, that is known to activate the IMD pathway, led to a reduction in lifespan and a generally high fitness cost, as expected. However, when the IMD pathway was activated for only a few days at a time by PGRP-LE under the GeneSwitch-inducible GAL4 system and feeding the flies on food supplemented with mifepristone (RU486), the lifespan and other fitness parameters was not reduced and the flies behaved normally, as assessed by measuring their heat tolerance, geotaxis and reproductive ability. Also, contrary to the previous infection-based assays of *Drosophila* fitness, flies with transiently activated immune responses survived significantly longer after infection with *Pseudomonas aeruginosa* Migula than their conspecifics lacking immune up-regulation (Libert *et al.* 2006). This result indicates that much of the negative fitness effect observed in the previously mentioned studies was likely due to the presence of the bacteria and a long-term immune activation, and not a transient transgenic immune response. If there were a cost to simply activating the immune system on a short time scale, flies with the inducible PGRP-LE would display a fitness cost similar to that of flies with constitutive expression, and they would not exhibit an increased lifespan after infection with various bacteria. Therefore, while immune activation certainly can lead to manifold negative fitness effects, there is also evidence that tissue-specific, short-term immune deployment is not detrimental to the insect's lifespan and may lead to a fitness advantage under certain conditions of infection.

Impact of immune response and transgene expression on mosquito fitness

While most research into the evolutionary costs of increased immune deployment has been performed in *Drosophila*, the results are only as relevant as the model organism employed: while *Drosophila* serves as a valuable genetic model, there are many differences between flies and important disease vectors. The fact that mosquitoes and other vectors of human disease are haematophagous adds a new dimension of complexity to their fitness, given that a blood meal may provide sufficient nutrients to make up for any reallocation of resources for the purpose of producing immune effectors. Conversely, the acquisition and digestion of a blood meal both require significant energy expenditure, given the challenge of finding a suitable host, breaking down the blood proteins to useable units and dealing with the many toxic compounds produced during blood digestion, such as heme and reactive oxygen species (Zhou *et al.* 2007). This necessary energy usage may only compound any energy shortages caused by immune deployment, again leading to a complex and somewhat unpredictable set of interactions that will affect the fitness of mosquitoes that are found or created to be refractory to disease transmission.

There is, however, evidence of a potential effect of immune activation on mosquito fitness, similar to that observed in *Drosophila*. A number of studies have indicated that infection of *Anopheles* mosquitoes with *Plasmodium* parasites reduces the lifespan and reproductive output of the mosquitoes (Anderson *et al.* 2000, Hogg and Hurd 1995). *Ae. aegypti* adults selected to be resistant to *P. gallinaceum* are significantly smaller, lay fewer eggs, and have shorter lifespans than susceptible conspecifics (Yan *et al.* 1997). These differences are not unexpected, given that similar results have been observed in *Drosophila* and because there is significant conservation between the *Drosophila* and mosquito immune systems (Christophides *et al.* 2002). Conversely, male *An. gambiae* from a line selected for increased melanotic encapsulation of *Plasmodium yoelii* show an increase in fecundity, as measured by the number of offspring born to their mates (Voordouw *et al.* 2008). Similarly, Dong *et al.* (2011) observed that transgenic mosquitoes that over-express the Rel2 transgene upon induction of the carboxypeptidase promoter following a bloodmeal have no reduction in longevity when fed only on sugar. Mosquitoes provided with a naïve blood meal likewise showed no reduction in longevity, while mosquitoes fed upon *P. falciparum* infected blood exhibited a minor reduction in lifespan, as well as a modest reduction in the number of eggs laid (Dong *et al.* 2011). These studies, taken together, show that an increased immune activity in mosquitoes may have disparate fitness effects, depending on the host-pathogen system, and that not all effects are negative. Also, careful measurement of the fitness costs imposed on the mosquito by both infection with *P. yoelii* and resistance showed that increased melanotic encapsulation of parasites has the same cost; both in terms of lifespan reduction and egg hatch rate (Hurd *et al.* 2005). Thus, a moderate fitness cost resulting from increased immune activation may be acceptable, since it will simply offset the fitness cost of infection.

In addition to any fitness effects caused by immune activation in mosquitoes, there may be effects related to genetic manipulation itself (Marrelli *et al.* 2006). Transgenesis allows the introduction of novel genes that can lead to refractoriness and also allows transient immune activation instead of constitutive up-regulation, which can limit any negative effects of immune over-expression, as discussed above. However, the creation of transgenic mosquitoes can carry with it an inherent cost to the transformed insect. Genetically modified mosquitoes made to constitutively express a green fluorescent protein after insertion with the *piggybac* transposable element have a competitive disadvantage when compared to both wild-type and inbred, but not transgenic, mosquitoes when reared together (Koenraadt *et al.* 2010). The negative effects of transgenesis were only compounded when limited food resources are provided and the adult transgenic mosquitoes have fewer energy reserves available. Thus, exogenous gene expression utilizes energy that would otherwise be used for development (Koenraadt *et al.* 2010). In a separate study, Amenya *et al.* (2010) created mosquitoes expressing an enhanced cyan fluorescent protein inserted into an *attP* docking site and saw no decrease in transgenic mosquito lifespan or fecundity (Amenya *et al.* 2010). Use of an *attP* docking site takes advantage of the site-specific integration of the φC31 integrase to insert transgenes into a known chromosomal location (Nimmo *et al.* 2006). By doing so, different transgenic lines can be created with the gene of interest inserted into a position with known fitness effect, allowing both minimization of negative fitness effects and the measurement and comparison of the effects of different transgenes on fitness independent of effects due directly to insertion. Li *et al.* (2008) observed no measurable effect on the adult survivorship, egg hatch rate or larval-to-pupal viability in *An. stephensi* mosquitoes that express the exogenous peptide SM1 under the carboxypeptidase promoter. However, during the same study, when the authors kept cages containing both transgenic and wild-type mosquitoes for multiple generations, they noticed that the frequency of genetically modified mosquitoes decreased over time. They attributed this effect to a lower reproductive capability of the transgenic mosquitoes or a negative consequence of the insertional mutagenesis, and not the expression of the transgene (Li *et al.* 2008). The same

group also observed that the transgenic mosquito line expressing SM1 has a fitness advantage over wild-type conspecifics upon infection with *P. berghei* (Marelli *et al.* 2007). This effect was seen not only in the form of a higher fecundity and longer lifespan in one generation, but also in the gradual replacement of wild-type mosquitoes by the genetically modified mosquitoes over multiple generations when fed on *P. berghei*-infected mice, but not when fed on uninfected mice (Marrelli *et al.* 2007). Taken together, these studies show that any effects of transgenesis on the mosquitoes will depend on the environment in which the mosquitoes live. These types of effects can be avoided by selection of the most fit transgenic lines after many have been created; however, if the effects are only slight reductions in lifespan or fecundity, they may not be noticed during the selection process. New methods of transgenesis that allow site-specific integration of the transgenes have recently been developed, allowing the selection of the insertion location and minimization of gene disruption (Amenya *et al.* 2010, Labbe *et al.* 2010, Meredith *et al.* 2011). However, it is more likely that any transgene introduced into the mosquito will lead to effects that reflect a reallocation of resources to producing the transgene, as previously described for immune activation. Also, an inserted gene may have more widely ranging effects than initially predicted, potentially leading to greater resource use or significant changes in gene expression. For instance, if an inducible transgene that affects both immune and developmental functions is introduced, the result may be a differential expression of numerous genes beyond the initially targeted immune genes, and therefore widespread effects on the mosquito and a greater fitness cost. Such effects, however, can be minimized by carefully selecting the gene to be introduced, expressing it in a highly tissue- and stage-specific manner and creating multiple transgenic lines, then monitoring and selecting the line with the least observable effect on lifespan, fecundity and other fitness measures before the insects are released. When multiple transgenic lines with the same transgene are created through random integration, both the expression of the transgene and the effects of integration on other genes can vary greatly. This variability is the result of position effects, i.e. variability in the expression of a gene that is a consequence of its location on the chromosome, and therefore its relative proximity to other genes or regulatory elements that act on all genes within their reach (reviewed in Wilson *et al.* 1990). Furthermore, the effects of the transgene on neighbouring genes will vary greatly depending on its final location: whether it has interrupted a gene or a regulatory sequence, or the interactions between the two. Thanks to our extensive knowledge of these effects, careful design of transgene constructs and selection of transgenic strains can minimize these effects of transgenesis.

It is also important to note that a small decrease in the fitness of a vector as a result of increased immune deployment or transgenesis would not preclude using this system as a vector-borne disease control technique. As discussed above, the vectorial capacity of an insect vector depends on numerous factors, including both the vector competence and daily survivorship of the vector. Activation of a specific arm of the innate immune system so as to reduce the ability of the mosquito to transmit a pathogen is, in effect, decreasing the vector competence of the mosquito and producing a related decrease in vectorial capacity. However, a decrease in daily survivorship, such as one caused by a fitness cost associated with gene expression, will also decrease vectorial capacity. Likewise, a decrease in survivorship or in the number of eggs produced by each generation will lower the mosquito density relative to human hosts, yet another factor that can lead to an overall reduction in vectorial capacity. Overall, a slight compromise in mosquito fitness leading to decreased fecundity and lifespan can lead to a large decrease in vectorial capacity, especially if combined with an additional reduction in vector competence. However, any decreases in fitness must be limited in scope so that they do not prohibit the genetically-modified insect from invading the natural population and maintaining a normal population; otherwise, the modified mosquitoes will never reach high enough numbers to be a viable tool for vector control. Use of