The effects of genetic modification on *Anopheles stephensi*

by

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A dissertation submitted to the Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

December 2014

Abstract

Various species of mosquitoes from the genus *Anopheles* vector the *Plasmodium* spp. parasites responsible for human malaria. While traditional, drug and insecticide based, efforts to control malaria have met with some success in limited geographic areas, there are still millions of cases each year and novel control strategies are needed to effect permanent reductions on a worldwide scale. One potential method to control malaria is to harness the mosquito immune system to create mosquitoes refractory to *Plasmodium* infection via genetic engineering. Efforts to create such mosquitoes have succeeded in multiple laboratories, but no such mosquitoes have been released as part of a large-scale malaria control program, partly due to a lack of knowledge about their biology. We undertook to expand our knowledge of the effects of genetic modification on *An. stephensi* mosquitoes. First, we characterized the transcriptomic and proteomic effects of transient up-regulation of the IMD pathway associated NF-kB transcription factor Rel2, showing that a very large number of both immune and non-immune genes are controlled by this up-regulation, and allowing us to identify novel anti-*Plasmodium* factors in the mosquito. We then measured the fitness of 5 genetically modified *An. stephensi* strains under a variety of conditions and using varied measures. These data showed that, while some genetically modified mosquitoes do bear a fitness cost due to the genetic modification, there is no inherent fitness cost to transgenesis. This indicates that genetically modified mosquitoes are a viable tool for malaria control and further efforts should pursue the testing of these mosquitoes on a larger scale in order to prepare them for use in global malaria control efforts.

Acknowledgments

I would like to begin by thanking my thesis advisor, Dr. George Dimopoulos, for allowing me to perform my research in his laboratory. Similarly, I would like to thank my thesis committee members, the Drs. Marcelo Jacobs-Lorena, Anna Durbin and Erika Matunis, as well as my alternates, the Drs. Doug Norris and Daniella Drummond-Barbosa, for their helpful comments. I would not have been able to perform this research without the help of Drs. Yuemei Dong, Chris Cirimotich and Simone Sandiford, or Messers Ben Blumberg and Anthony Gacita and Mrs. Alekhya Dhawan who both instructed me in the laboratory and helped me perform numerous experiments. Likewise, I would like to thank all the other members of the Dimopoulos group for their help and friendship. Thank you to the entire departmental staff of MMI and the JHMRI for their help keeping me on track to graduate, most especially Gail O'Connor, Maryann Smith and Genevieve Williams. Funding for various projects was supplied by the JHMRI, TMDC of Baltimore, the JHSPH GHFRA and the NIH, without which none of this would have been possible. Finally, thank you to all my family and friends, too many to list here, for everything they've done for me. I would especially like to thank my parents and sister, my Jose's family, the bartenders at Bad Decisions, Max's and all the other great Baltimore bars, the head distiller of Knob Creek, and my fiancée Alexandra, for everything they've done for me.

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(Modified from Pike, A., C. Cirimotich, and G. Dimopoulos. 2013. Impact of Transgenic Immune Deployment on Mosquito Fitness. *In* W. Takken and C. J. Koenraadt [eds.], Ecology of Parasite-Vector Interactions.Wageningen Academic Publishers, Wageningen)

1.1 Introduction

Mosquitoes vector a variety of important human pathogens including viruses, filaria and apicomplexan parasites. Mosquitoes acquire these infections while taking a blood meal necessary for reproduction, and pass them on when they take subsequent blood meals. While traditional vector-control methods have been used to control these diseases for many years, they have yet to make lasting inroads in the battle to eliminate diseases such as malaria and dengue fever. Tools such as insecticides, bed nets and larval source management are able to reduce infections in an area temporarily; however, constant application of these mosquito control methods is necessary, as the diseases rebound soon after control measures are removed (Smith et al. 2013a). Due to this, in order for permanent removal of vector-borne diseases to be achieved, new technologies must be developed that can effect lasting change in vector populations or behaviors and eliminate the diseases.

A variety of factors contribute to the ability of a mosquito to transmit a pathogen successfully 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 (Ross 1911) and later refined by Macdonald (Macdonald 1957) and Smith and MacKenzie (Smith and McKenzie 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, 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.

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. This new technology has led to significant advances in our understanding of how the mosquito's innate immune system is actively involved in eliminating large fractions of these human pathogens, sometimes rendering the mosquito vector completely refractory to infection (Garver et al. 2009). 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 super-immune mosquitoes (Dong et al. 2011). 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.

Two main mosquito populations have been targeted for genetic modification. The yellow fever mosquito, *Aedes aegypti*, is the main vector for urban dengue fever. With a wide geographic range, this mosquito, along with the closely related *Ae. albopictus*,

spread dengue virus and various other viruses to people around the world (WHO 2014a). Due to its preference for living in urban areas and biting humans, *Ae. aegypti* is an efficient vector of dengue virus and leads to large numbers of infections each year. While *Ae. albopictus* also acts as a vector for dengue virus, *Ae. aegypti* is considered the main vector of the disease and is likely responsible for the majority of the 50-100 million cases each year from dengue virus (WHO 2014a). The mosquito has also been moving around the world rapidly due to human movement, leading to the possibility of this disease traveling to new geographic areas or returning to places after the disease has been removed, as new vector populations can become established and start spreading the disease (Brown et al. 2014). Genetic modification of *Ae. aegypti* has occurred in multiple laboratories with the intent of using these mosquitoes to reduce dengue associated morbidity and mortality, and field trials with some of these strains are now underway (Bian et al. 2005, Franz et al. 2006).

Anopheles spp. mosquitoes have also been genetically modified in multiple laboratories (Ito et al. 2002, Dong et al. 2011, Isaacs et al. 2012). Various anopheline mosquitoes are the vectors for the *Plasmodium* spp. parasites responsible for human malaria, though the specific mosquito species responsible for transmission varies from area to area. Malaria causes upwards of 200 million cases and 650,000 deaths each year world-wide, largely among African children, and has been called the most important vector-borne disease affecting man (WHO 2014b). There have been many efforts over the years to curb malaria's death toll, however none has been able to cause a significant reduction in the hardest hit areas. While effective drugs to treat malaria cases exist, an effective vaccine has not yet been licensed and vector control through bednets and indoor

residual spraying remain the front line of defense against the disease. Therefore, genetic modification of anopheline mosquitoes may form an important and component of future malaria control programs. Two important species that have been well studied in the laboratory, have had their genomes sequenced and have been genetically modified in the laboratory successfully are *An. gambiae*, the primary malaria vector in much of Sub-Saharan Africa, and *An. stephensi*, an urban malaria vector from the Indian subcontinent (Holt et al. 2002, Jiang et al. 2014). While not as universal in their distribution and worldwide importance as malaria vectors as *Ae. aegypti* is for dengue virus, these two species are good representatives of the tribe and are a good pair to start with for proving the viability of using genetically modified mosquitoes to control malaria.

Despite the availability of tools to make genetically modified mosquitoes and multiple laboratories capable of doing that, no such tools have yet been used as part of a worldwide dengue or malaria control program. This stems from a variety of issues including differing views of how to use genetically modified mosquitoes to reduce disease incidence, potential negative fitness effects of genetic modification, public perception of genetically modified organisms as a whole and difficulties determining the necessary number of mosquitoes to release and the ability to produce those mosquitoes.

1.2 The mosquito innate immune system

Infection of a mosquito with a virus or parasite 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. 2006b, Xi et al. 2008b). 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 hemolymph 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 defense for the mosquito against a variety of invading pathogens. PRRs can also directly activate immune defense mechanisms such as phagocytosis and complement-like killing mechanisms, independent of the intracellular immune signaling pathways.

Various cellular and humoral factors in the mosquito hemolymph play a significant role in the response to microbial challenge. Circulating immune-competent cells, known as hemocytes, 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 signaling 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, Jiang et al. 2014), the three major immune signaling pathways (Toll, IMD, and Jak/Stat) that were originally described in *Drosophila* or mammals have been identified through orthology in mosquitoes (Christophides et al. 2002).

The Toll pathway has been implicated in the mosquito defense against fungal, bacterial, parasitic and viral infections (Shin et al. 2005, Xi et al. 2008b, Antonova et al. 2009). 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 *Ae. aegypti* genome also encodes two distinct Toll pathway transcription factors (REL1A and REL1B), while the *An. gambiae* 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* defense against dengue viruses (Xi et al. 2008b, Ramirez and Dimopoulos 2010) and the *An. gambiae* defense against rodent malaria parasites (Meister et al. 2005, Frolet et al. 2006, Zou et al. 2008, Garver et al. 2009). Frolet et al. (2006) 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. These authors 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, An. stephensi* and *An. albimanus*, as well as *P. gallinaceum* infection of *Ae. aegypti* appear to be controlled through Toll pathway activation, while *P. falciparum* infection of various anopheline mosquitoes is affected to a lesser degree by the Toll pathway (Zou et al. 2008, Garver et al. 2009).

Initiation of signaling through a second innate immune pathway, the immune deficiency (IMD) pathway, protects mosquitoes from infection with the human malaria parasite *P. falciparum* (Meister et al. 2005, Garver et al. 2009, Dong et al. 2012). Signaling 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 (Meister et al. 2005, Luna et al. 2006). 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 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 late stage *Plasmodium* infection in *Anopheles* spp. (Gupta et al. 2009). 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. Recently, the pathway has been shown to mediate the killing of *P. falciparum* and *P. berghei* parasites after midgut invasion (Gupta et al. 2009). 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* mosquitoes (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 (Cirimotich et al. 2009). Of particular interest is the thioester-containing protein TEP1, which has been shown to be crucial for mosquito defense against *Plasmodium* parasites (Levashina et al. 2001). TEP1, a homolog to the vertebrate complement system molecule C3, is constitutively secreted by hemocytes into the mosquito hemolymph, 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 (Levashina et al. 2001, Blandin et al. 2004, Garver et al. 2009).

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.

1.3 Mosquito transgenesis

The introduction of novel genetic elements into mosquito genomes has become a powerful system 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 and makes it possible to express the gene 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. 1996, 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, and new strains of additional species continue to be made.

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 (Shin et al. 2003, Antonova et al. 2009) and that RNA interference is crucial for antiviral defense (Khoo et al. 2010). 2010). Similarly, Rel-2 overexpressing *An. stephensi* mosquitoes have been used to profile the global transcriptomic and proteomic changes brought about by Rel-2 induction, and have identified Rel-2 both as controlling the mosquito defense against *P. falciparum*, as well as driving expression of numerous immune and non-immune genes. Transgenesis has also been used in both *Aedes* and *Anopheles* mosquitoes to express heterologous genes for the purpose of altering vector competence (Cirimotich 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 (Franz et al. 2006, Mathur et al. 2010, Isaacs et al. 2011). 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 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 *Ae. aegypti* to demonstrate that this principle may eventually be applied to the engineering of pathogen-resistant mosquito populations. 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 hemolymph (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 cecropin A or defensin A, 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).

Similarly, the first and second strategies have been pursued in *An. stephensi* mosquitoes, and the first in *An. gambiae*. As in *Ae. aegypti*, mosquitoes of these two species have been made that overexpress both their own immune genes as well as novel, non-native, anti-*Plasmodium* effectors such as single-chain antibodies and various toxins linked to pathogen recognition motifs (Ito et al. 2002, Dong et al. 2011, Dong et al. 2012, Isaacs et al. 2012). These mosquitoes have been shown to have greatly increased resistance to infection with the human malaria parasite, *P. falciparum*, as well as alter the expression of large and varied gene families, both with and without known immune function (Pike et al. 2014). Also as in *Aedes* mosquitoes, these effects were seen under different promoters, indicating that the same gene expressed at different times or in different tissues can have a profound effect on *Plasmodium* infection levels (Dong et al. 2011).

While manipulation of the mosquito immune system allows a variety of ways to target human pathogens in the vectors, there are also a variety of non-mosquito constructs that can lead to the same result (Isaacs et al. 2012). The use of toxins and antibodies from other organisms creates a variety of killing mechanisms not found within mosquitoes and which the pathogens will not have encountered before, and against which they will not, therefore, have natural resistance. In addition, multiple of these killing mechanisms could be combined into one mosquito, or combined with mosquitoes bearing enhanced immune systems, leading to mosquitoes that are more resistant to the pathogens and which reduce the possibility of the development of parasite resistance to the defense mechanisms.

However, these additional mechanisms are truly transgenic (based on non-mosquito genes) and may be met with increased public resistance when released.

In addition to using genetic modification to increase the expression of immune genes to fight pathogen infection, genetically modified mosquitoes have also been made that suppress the natural population to reduce the number of infectious bites. Reduction of pest populations has formed a component of the management of numerous insect species, both in agriculture and public health, and has been aided by genetic tools. The sterile insect technique (SIT) has been used for decades to reduce pest populations successfully, including in projects such as screwworm eradication in many areas and reductions in mosquito populations for disease control (Wyss 2000, Oliva et al. 2013a). Traditionally, SIT has depended on radiation or chemical based sterilization of male mosquitoes that are then released to mate wild-type females. However, these methods have serious drawbacks such as reduced male fitness and mating competition, as well as needing the constant rearing of large numbers of mosquitoes for release. Creating genetically modified mosquitoes for release of insects with a dominant lethal gene (RIDL) has been proposed and initial experiments have been carried out as an alternative to these traditional methods (Phuc et al. 2007).

RIDL depends on the use of male mosquitoes carrying a dominant lethal gene. This gene will kill all offspring of a mating between the genetically modified male and any female mosquito (Alphey et al. 2013). Mosquitoes would be reared in large numbers, the females would be removed and the males released into the wild where they would mate with wild-type females. Any offspring from these matings would not be viable, causing a decrease in the population of mosquitoes and decreasing local disease

transmission. Mosquitoes such as this have been created by Oxitec, and experimental releases have begun in multiple locations around the world (Phuc et al. 2007). Oxitec has tested limited releases of their OX513A strain of RIDL mosquitoes in both the Cayman Islands and Malaysia, and company releases report an 80% decrease in *Ae. aegypti* populations following the release of 3 million genetically modified mosquitoes in the Cayman Islands (Harris et al. 2012). Further studies are ongoing in Brazil to better determine the necessary release rates and conditions, and other sites are being considered for limited or widespread releases (Oxitec.com 2014).

While RIDL mosquitoes have been created and are being tested, there are drawbacks to this approach. First, for the method to be effective, a high proportion of the matings must result in sterility, and the genetically modified mosquitoes must be able to compete with the wild-type males. In cage trials, the OX513A line shows similar mating success to wild-type males and nearly 100% sterility, but this may not hold true in the field (Bargielowski et al. 2011, Massonnet-Bruneel et al. 2013). In Oxitec mosquitoes, the population is maintained in the laboratory by controlling the lethal gene with a repressible promoter that is turned off in the presence of tetracycline (Phuc et al. 2007). When larvae from incompatible matings are maintained on a diet containing tetracycline, they live, overcoming the lethality. However, the same could occur in the wild if tetracycline is found in natural water sources. Tetracycline is a commonly used antibiotic on both farms and for human disease, and therefore may be present in runoff. Both the amount of tetracycline in natural mosquito habitats and the amount necessary to repress expression of the lethal gene need to be determined in order to understand if this will be a problem for widespread releases.

There are potential ecological consequences to removing mosquitoes from an area, which need to be investigated further. However, these concerns also exist with current insecticide based mosquito control methods, and RIDL may be superior as there will be fewer off-target effects of releasing these mosquitoes than using broad-spectrum insecticides. It is also possible that mosquitoes in the field would evolve to survive the lethal gene, rendering the intervention ineffective. Traditional sterilization methods based on radiation or chemical treatment damage the DNA in many random areas at once, making evolution to avoid the damage difficult, whereas RIDL targets a single DNA location each time, making evolution away from that sequence possible (Oliva et al. 2013a). This is a potential problem with all interventions, including traditional control means, and can be overcome by developing other dominant lethal genes in the mosquitoes for subsequent releases if needed, efforts toward with are already underway.

In order for population replacement or suppression to succeed, the mosquitoes must be able to successfully mate with the wild-type population at a high level or in its entirety. Any significant number of wild-type mosquitoes left in an area with high levels of transmission indicated by a high entomological inoculation rate will lead to continued transmission in the area. The ability of mosquitoes to replace or suppress the wild-type population successfully depends on the fitness costs of the genetically modified mosquitoes relative to their wild-type conspecifics, the size of the original population, and the existence of a gene driver, as well as logistical issues related to rearing and releasing large numbers of mosquitoes (Boete and Koella 2002).

1.4 Fitness of genetically modified mosquitoes

It has generally been assumed that the genetic modification of any organism will lead to a decrease in the fitness of that organism. This is especially true when discussing modifications meant to increase the immune activity of the organism (Marrelli et al. 2006). Part of this assumption is based on early studies of immunity that showed a fitness cost, and part depends on the assumption that if increased immunity were not detrimental it would have evolved on its own, both of which are flawed arguments (DeVeale et al. 2004). Early studies on the effects of insect immunity on fitness were largely performed in *Drosophila*, and often depended on infecting the fly with a pathogen and observing the fitness effects (McKean and Nunney 2001, McKean et al. 2008, Imroze and Prasad 2012). For instance, injecting a large number of bacteria directly into the hemocoel of *Drosophila* was shown to lead to a significantly shorter lifespan and fewer offspring, especially in food-limited situations (Bedhomme et al. 2004, Zerofsky et al. 2005). Similarly, flies that are able to fight off invasion by the parasitoid was *Acyrthosiphon pisum* show reduced size and fecundity, again potentially due to immune activation (Kraaijeveld et al. 2002). However, studies of this type ignore the effects of the bacterial infection and injury on the fly. An injection such as that will create a systemic sepsis that is likely to have effects on the fly that go far beyond those caused by the immune system itself.

Likewise, the assumption that increased immunity would have evolved on its own if there were no fitness cost to doing so assumes that there is a fitness cost to infection with the pathogen. If pathogens have no fitness cost prior to the first batch of offspring there will be no selective pressure leading to a stronger immune system, regardless of any fitness cost of immunity or lack thereof. Pathogens that infect mosquitoes, especially

human pathogens, often have an incubation period of three or more days before reaching high numbers, during which time the female is able to lay her first batch of eggs. Therefore, even if the pathogen does cause a fitness cost it will not take effect until after reproduction has happened, and will not put pressure on the mosquito to evolve increased immunity.

While there has been significant research into the evolutionary costs of increased immune deployment in *Drosophila*, similar studies in mosquitoes are limited and the results of *Drosophila* studies 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 hematophagous 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 creating 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.

Multiple groups have begun to study the fitness of genetically modified mosquitoes under a variety of conditions (Catteruccia et al. 2003, Irvin et al. 2004). Initial experiments in this regard appeared to corroborate the assumption that there would be a

fitness effect of genetic modification. However, these experiments were done in lines with constitutive activation of the inserted gene, which may have been very energetically costly (Catteruccia et al. 2003, Irvin et al. 2004). More recent creation of genetically modified mosquitoes, whether based on upregulation of their own immune systems or with genes from other organisms inserted, has focused on transient immune activation to ameliorate these effects (Moreira et al. 2004). To achieve this, blood-meal inducible promoters such as the vitellogenin and carboxypeptidase promoters have been used. Genes under the control of both of these promoters are upregulated soon after a blood meal is consumed and return to baseline levels within two days (Dong et al. 2011).

There is, however, evidence of a potential effect of immune activation on mosquito fitness, as has been 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 (Hogg and Hurd 1995, Anderson et al. 2000). *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 *P. yoelii* show an increase in fecundity, as measured by the number of offspring born to their mates (Voordouw et al. 2008). These studies, taken together, indicate 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 indicated 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.

More recent studies with genetically modified mosquitoes transiently overexpressing immune genes have shown little or no reduction in lifespan, fecundity or size in multiple laboratory experiments, and have even been able to persist during mixed-cage trials (Dong et al. 2011, Smith et al. 2013b). In mixed-cage trials with wild-type mosquitoes, these genetically modified mosquitoes have actually been able to increase in prevalence when maintained on *P. berghei* infected blood at each generation (Smith et al. 2013b). While large-scale cage and field trials must be done to ensure this effect stays true for larger populations, these recent results show no reason to believe there is a significant fitness cost associated with transient immune deployment. This may be due to the fact that mosquitoes have an abundance of available protein immediately after a blood meal, allowing them to expend extra energy on overexpressing the immune genes during the time they are active. By the time the blood-meal based energy is depleted the expression of the gene has returned to normal and no extra energy is needed for egg-laying and general living. Similarly, during the time of gene overexpression the mosquitoes will largely be resting to digest the blood meal and may not need that much energy.

In addition to any fitness effects caused by immune activation in mosquitoes, there may be effects related to genetic manipulation (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 express green fluorescent protein constitutively 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). However, the negative effects of transgenesis are 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, 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 protein 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). Conversely, the same group also observed that the transgenic mosquito line expressing SM1 has a fitness advantage over wild-type conspecifics after infection with *P. berghei* (Marrelli et al. 2007). This effect was seen not only in the form of a higher fecundity and longer lifespan after infection 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). Similar effects were found with mosquitoes bearing a phospholipase A(2) gene and maintained on *P. falciparum* infected blood, though these studies showed that the effects depended on the genomic insertion location of the gene (Smith et al. 2013b). These studies indicate that any effects of transgenesis on the mosquitoes will depend on the environment in which the mosquitoes live and the genomic integration area of the transgene. 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 (Labbe et al. 2010, Meredith et al. 2013). However, it is more likely is 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 as has been shown in *An. stephensi*, 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. If multiple transgenic lines are created with the same insert 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 (Wilson et al. 1990). Furthermore, the effects of the transgene on neighboring 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 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 a decrease in the number of eggs produced by each generation may lower the mosquito density relative to human hosts, yet

another factor that can lead to an overall decrease in vectorial capacity. Overall, a slight decrease in mosquito fitness leading to decreased fecundity and lifespan can lead to a large decrease in vectorial capacity, especially if combined with an additional decrease in vector competence. However, any decreases in fitness must be limited in scope so that they do not prohibit the vector 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 strong gene drivers, such as homing endonucleases or medea elements, should be able to mitigate some of the fitness cost and still drive the genes of interest into the natural population (Cirimotich et al. 2011). This goal can be met by increasing the probability that a gene will be spread to the mosquitoes' offspring or by giving mosquitoes bearing the gene driver a significant fitness advantage over those that lack the gene driver, such as by killing offspring that lack the driver. As long as the transgene being introduced into the mosquito population is linked to the gene driver, it will be carried with the driver, and the genetically modified mosquitoes will replace the wild-type population to a transgenic population. However, if the fitness cost is too high, even a strong gene driver will not be able to overcome the negative effects of transgenesis, and the population will not be replaced.

Numerous studies have also been performed on the fitness of RIDL strains of mosquitoes. These studies focus on the mating ability of the males, as this strategy is based on the release of large numbers of males that will mate with the wild-type females, causing incompatibility (Bargielowski et al. 2011, Massonnet-Bruneel et al. 2013). Similar strategies have been successfully employed to reduce crop pests using SIT or

mosquitoes using *Wolbachia* infected mosquitoes, and the fitness of RIDL strains must be evaluated in comparison to these previous efforts (Laven 1967, Wyss 2000). SIT is accomplished by either chemically sterilizing or irradiating the males, which leads to a decrease in fitness due to the large amounts of damage done during sterilization (Oliva et al. 2013b, Yamada et al. 2014). Reduced application of the chemosterilant or radiation can lead to less fitness cost, but this can also lead to an increased number of non-sterilized mosquitoes being released, which reduces the efficacy of the program. RIDL males have been engineered and selected to have minimal fitness effects, though some small fitness effects have been observed (Bargielowski et al. 2011, Massonnet-Bruneel et al. 2013). This, however, is less than the effect caused by irradiation, though more studies are necessary to determine the full effects of this minimal fitness cost in the field.

1.5 Logistics of genetically modified mosquito deployment

In order to use genetically modified mosquitoes as part of a malaria control program the proper release ratios must be established, the mosquitoes must be reared in appropriate numbers and those mosquitoes must be released in the correct areas. Many models have been created to determine the proper release ratios of genetically modified mosquitoes for both population replacement and RIDL(Boete and Koella 2002, 2003, Koella and Boete 2003). In order for population replacement to be effective in an area with sustained malaria transmission the population must be completely replaced, as even a few remaining wild-type mosquitoes(Alphey et al. 2011) may be able to keep the transmission cycle going (Boete and Koella 2002). In order to make this happen, the fitness costs must be minimal, a goal which is in sight. Additionally, either a gene driver

must be used or the population must be suppressed to the point at which the genetically modified mosquitoes overwhelm the wild-type population (Marshall 2009, Robert et al. 2013). 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. Gene drivers, such as transposable elements, medea-like elements and homing endonucleases have been suggested for use in this regard, but have yet to be created for wild-type mosquitoes (Sinkins and Gould 2006, Chen et al. 2007, Windbichler et al. 2011). The bacterium *Wolbachia* has also been suggested as a gene driver and has recently been introduced into *An. stephensi* mosquitoes, but this introduction causes a large decrease in fecundity in the mosquitoes, which may hamper its usefulness (Walker and Moreira 2011, Bian et al. 2013, Joshi et al. 2014). Further, no one has investigated the use of *Wolbachia* in this way, and it is possible that the gene would become unlinked from the infection, rendering the driver ineffective. Alternatively, models suggest that population suppression followed by inundative releases may eliminate the need for such a gene driver, but no large-scale field trials have yet shown this to be an effective method (Robert et al. 2013). The necessary release ratios for RIDL mosquito releases have also been modeled, and experiments are ongoing now with the OX513A Oxitec line to determine if these estimates are correct and further refine them under field conditions (Alphey et al. 2011).

Once the release ratios for genetically modified mosquitoes are established, the mosquitoes must be reared and released. While many insectaries operate worldwide, most cannot rear mosquitoes at the scale necessary to maintain this sort of intervention. However, advancements in both rearing techniques and technologies for sorting

transgenic from wild-type larvae have advanced to the stage at which large-scale rearing is possible (Balestrino et al. 2012, Marois et al. 2012). The OX513A Oxitec line is being reared in very large numbers close to sites for release, indicating that similar efforts would be successful in other areas (Harris et al. 2012). After rearing, mosquitoes must be released over a large area in order for them to mate with as many wild-type conspecifics as possible, regardless of which strategy is being employed (Kiszewski and Spielman 1998). This sort of release has been accomplished in other insect species for control and could easily be adapted to mosquito releases (Wyss 2000). Engaging the public is one possible way to increase the distribution area, and may serve to help target mosquitoes to the areas occupied by humans, reducing incidence in those areas more quickly.

1.6 Public perception of genetically modified mosquitoes

Even if all other obstacles were overcome and a genetically modified mosquito strain was ready for worldwide deployment today, public perception of the releases would be necessary to assess. Many people, both in the developing and developed world, are highly resistant to genetically modified organisms in general, and may, therefore, be opposed to the idea of releasing genetically modified mosquitoes. This often stems from a misunderstanding of the genetic modifications and can hamper the employment of highly useful strains, both in agriculture and in other settings. By engaging the public and educating them both about the methods used to create the genetically modified mosquitoes and how these strains can help them they may be more willing to accept them (Subramaniam et al. 2012). Similar efforts have been necessary for past mosquito releases with non-genetically modified mosquitoes, and have met with success (Laven

1967). Studies of public perception also indicate that people may not resist this type of release if it is meant to benefit them (Amin and Hashim 2013, De Freece et al. 2014). The scourge of malaria is great enough that a long-term intervention such as replacing or removing the mosquito population will be popular, especially once successfully implemented in one area. OX513A Oxitec releases have occurred in three separate countries and have not met with excessive public resistance, and there is hope that expanded use of this strain or further releases with other strains would meet with similar acceptance (Subramaniam et al. 2012).

1.7 Conclusions

Due to the failures of previous vector-control efforts to remove mosquito-borne diseases from many areas, new tools such as genetically modified mosquitoes are increasingly necessary. Strains to be used for both population replacement and population suppression have been developed in laboratories, and evaluation of their usefulness for vector-control in the field is ongoing. While the two strategies differ in many ways, the components necessary for their success are similar. The mosquitoes must be fit enough to compete with their wild-type conspecifics, reared in large numbers and released over a large area to be successful, solutions to all of which are currently being investigated. These experimental results, along with the ongoing releases of Ox513A Oxitec strain mosquitoes in Brazil indicate that genetically modified mosquitoes may soon become a part of global vector-control efforts. Continued research will only yield improvements in this regard, but large-scale field trials should be pursued to advance the field. The

potential usefulness of such mosquitoes cannot be understated, and implementation of these control strategies should be pursued as soon as safely possible.

Chapter 2: Characterization of the Rel2-regulated transcriptome and proteome of *Anopheles stephensi* identifies new anti-*Plasmodium* factors.

(Published September 2014 in Insect Biochemistry and Molecular Biology 52:82-93)

2.1 Introduction

Mosquitoes are vectors for many important human pathogens, including viruses, filarial worms, and apicomplexan parasites. A number of *Plasmodium* spp. parasites, vectored exclusively by *Anopheles* spp. mosquitoes, cause human malaria. Because of difficulties in the distribution of anti-malarial chemotherapeutics and the rise of drug resistance in the parasite, vector control remains at the forefront of malaria control efforts. However, after decades of insecticide spraying, bed net distribution, and habitat remodeling, the disease remains established, so novel vector-control methods must be developed. Recently, methods have been developed to generate genetically modified mosquitoes (Ito et al. 2002), and various strategies based on their release are being investigated for malaria control. Conversion of a natural mosquito population to a transgenic population that overexpresses anti-*Plasmodium* immune system activators or effector molecules could represent one such method, and multiple mosquito lines expressing such transgenes in different tissues have already been developed (Dong et al. 2011, Dong et al. 2012).

Mosquitoes possess an innate immune system that is capable of responding to, and controlling, infection by diverse pathogens, including bacteria, viruses, and apicomplexan parasites (Cirimotich et al. 2009, Garver et al. 2009). Two immune pathways, the Toll and immune deficiency (IMD) pathways, have been extensively studied in both *Drosophila* and mosquitoes. Both pathways recognize invading

pathogens through the association of host pattern recognition receptors (PRR) with pathogen associated molecular patterns (PAMPs), leading to a signaling cascade, nuclear localization of transcription factors, and subsequent induction of the expression of numerous immune effector molecules and anti-microbial peptides. Invading pathogens are then killed by various mechanisms, such as phagocytosis and complement-like killing.

The nuclear translocation of the NF-κB transcription factor Rel2 leads to an induction of immune gene expression that constitutes the IMD pathway-mediated immune response (Meister et al. 2005). The IMD pathway has been shown to regulate the mosquito's resistance to *P. falciparum* infection (Garver et al. 2009), and numerous mosquito lines with inducible overexpression of the constitutively active short form of Rel2 have been created (Dong et al. 2011). One such line (henceforth referred to as the CP15 line) uses the carboxypeptidase gene promoter to limit Rel2 overexpression to the midgut following a blood meal, while another line (the VG1 line) overexpresses the same Rel2 transgene under the control of the vitellogenin gene promoter, leading to fat body-specific expression after a blood meal (Dong et al. 2011). Both these lines exhibit a greatly reduced susceptibility to *Plasmodium* infection following an infected blood meal and may represent viable tools for future release as part of a malaria control program. However, immune pathways and their downstream transcription factors can regulate a large variety of both immunity and non-immunity related processes (Dong et al. 2006a, Xi et al. 2008a). Hence, the overexpression of the Rel2 transcription factor affects the immune system, but it is also likely to regulate other physiological processes entailing genes of diverse functions.

A majority of studies on the insect immune system have relied on infection with a pathogen and observation of the insect's response to the insult (Zerofsky et al. 2005, McKean et al. 2008, Imroze and Prasad 2012). Using transgenic mosquitoes that overexpress Rel2 in an inducible fashion provides a pathogen-independent system to study IMD pathway-regulated immune response and eliminates any confounding factors brought about by the presence of the infecting organism. Zou and colleagues (Zou et al. 2011) used a Rel2-overexpressing *Aedes aegypti* to study the IMD pathway-regulated transcriptome.

We used whole-genome oligonucleotide microarrays to study recombinant Rel2-induced changes in mRNA abundance, as well as isobaric tags for relative and absolute quantitation (iTRAQ) to study changes in protein abundance after Rel2 overexpression in transgenic mosquitoes. Measuring the expression levels of both mRNA and protein allowed us to look for correlations between transcript and protein abundance following up-regulation of a transcription factor. We then used RNA interference (RNAi) assays to investigate a subset of genes, both with and without known immune function, for anti-*Plasmodium* and anti-bacterial activity, leading to the identification of multiple novel anti-*Plasmodium* effectors.

2.2 Materials and Methods

Mosquito rearing

A. stephensi Liston strain wild-type, CP, and VG transgenic Rel2-overexpressing lines (Dong et al. 2011) were maintained according to standard insectary procedures. In brief, larvae were reared at low densities in trays and fed a combination of ground fish flakes

(Tetra) and cat food pellets (Purina). Upon emergence, adults were maintained on a 12 h/12 h light/dark cycle at 27° C with 80% humidity and constant access to a 10% solution of sucrose in water. In order to stimulate egg production, adults were fed on ketamine-anesthetized mice according to IACUC-approved protocols.

RNA extraction and microarrays

One-week-old adult female mosquitoes were given a human blood meal from water-jacketed membrane feeders maintained at 37° C. Mosquito tissues were dissected in sterile PBS as follows: midguts were collected at 6 and 12 h after blood feeding, while fat bodies were collected at 12 and 18 h after the blood meal. Total mosquito RNA from dissected tissues was extracted using RNeasy kits (Qiagen) according to the manufacturer's protocols and quantified on a NanoDrop spectrophotometer before quality assessment on an Agilent Bioanalyzer 2100. Probes were synthesized using 200 ng of RNA and the Low-Input RNA Labeling Kit (Agilent) according to the manufacturer's protocol. These probes were hybridized to a custom-designed Agilent microarray slide, which was scanned with an Axon GenePix 4200AL scanner at 2-μm resolution. After scanning, statistical analysis was performed using the TIGR, MIDAS, and TMEV software packages (Dudoit et al. 2003), following standard laboratory protocol (Dong et al. 2006a), and analysis was performed using a *t*-test, with a significance level of α=0.05. Changes in gene expression were considered significant if the absolute value of the gene regulation was >=0.75 on a $\log_2$ scale. For each treatment, three biological replicates and one psuedoreplicate were performed. The array was designed using Array Designer software (Premier Biosoft, www.premierbiosoft.com) and based on an early version of

the *A. stephensi* transcriptome obtained from Dr. Jake Tu of Virginia Polytechnic Institute and State University, and putative function and gene ontology (GO) terms were assigned to transcript sequences based on homology to previously annotated *A. gambiae* genes discovered by a blastn search (Altschul et al. 1990). The blast search was performed against gene set AgamP3.7, downloaded from vectorbase.org; for each gene, the most significant hit was used for annotation, with a maximum e-value of 0.0001 used as a cutoff. Any genes that did not have significant homology to any previously annotated *An. gambiae* genes were used for a blastn (Altschul et al. 1990) search against the non-redundant nucleotide database from NCBI to assign putative function if similar genes or conserved sequences were identified in other species. While the gene with the highest blast homology between *An. stephensi* and *An. gambiae* may not represent a true orthologue, the early state of the annotation of the *An. stephensi* genome leaves this as our best prediction. Seven genes were analyzed by qRT-PCR to verify the results of the microarray (Figure 2.1).

Protein extraction and iTRAQ

One-week-old adult female mosquitoes from the WT, CP, and VG lines were given a human blood meal from membrane feeders at 37° C. Prior to the blood meal and 24 h afterward, mosquitoes were dissected in sterile PBS and their midguts and fat bodies collected. Three replicates of 10 midguts or fat bodies were resuspended in lysis buffer (10 mM HEPES, 0.1 mM $MgCl_2$, 0.1% Triton, and protease inhibitors [Roche]) and left on ice for 30 min. Tissues were homogenized, freeze-thawed twice, and centrifuged at

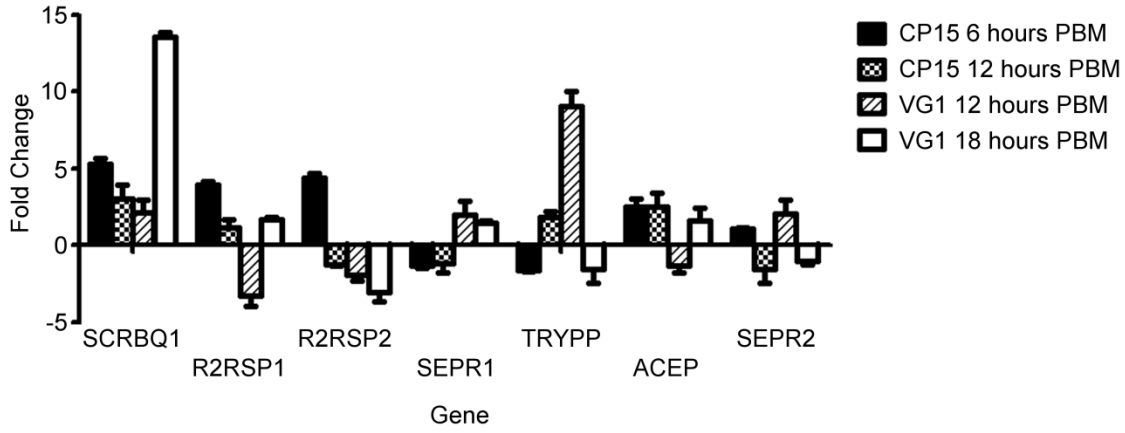

Figure 2.1: qRT-PCR verification of microarray data. 7 genes upregulated in the 12 hours PBM midguts samples of the microarray were chosen and their fold-change measured by qRT-PCR to verify their regulation. Of the 7, 4 were similarly regulated as measured by qRT-PCR and all 7 were upregulated in at least one experimental condition.

14,000 rpm for 30 min at 4°C. The supernatant fraction was collected and used as the total protein extract. Total protein concentration was measured by Bradford assay. Consistency among the three replicates was assessed by running 0.1 µg of total extract on a 4-12% Tris gel and silver staining. Replicates were then combined and used for iTRAQ analysis: 50 µg of protein was reduced using 2 µL of 50 mM TCEP for 1 h at 60° C, cooled to room temperature, and incubated with 1 µL of 100 mM MMTS for 15 min in the dark. Samples were then TCA-precipitated, and air-dried pellets were submitted to the Johns Hopkins School of Medicine Mass Spectrometry and Proteomics Facility for identification and relative quantitation using iTRAQ. The samples were trypsin-digested, labeled with 8plex iTRAQ reagents, fractionated by both strong cation exchange and reverse phase chromatography, and identified by mass spectrometry using a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) as described in detail (Ross et al. 2004, Guo et al. 2007, Martin et al. 2008, Pierce et al. 2008). MS/MS spectra were searched against a custom *A. stephensi* database generated from sequences provided by

Gene Name	Primer type	Primer sequences (Forward/reverse)
SCRBQ1	dsRNA	TAATACGACTCACTATAGGGCTGTTCGAAGCGATACTCC TAATACGACTCACTATAGGTGTAGTGCCAGATGCGTTTC
SCRBQ1	qPCR	CGGACAGGTGCGTGGATCGG GCAGGAAGAGGTTGAGCGGGG
AGBP1	dsRNA	TAATACGACTCACTATAGGCTGGTGCGTGATCTGAAGAA TAATACGACTCACTATAGGGGGAATGTTGGTGTACGGTC
AGBP1	qPCR	GCCCGAGTGCACCCCGAAAC CGCGATCCGCCCGATTCCAG
NPC2	dsRNA	TAATACGACTCACTATAGGTCCAATCTGGTCATTGCAGA TAATACGACTCACTATAGGCTTCAACTCCACCGTAAGGC
NPC2	qPCR	GGCATGTGCCCCGTATCCCG CCTGCAGCTGGAAGCAGGTAACG
A2MRAP	dsRNA	TAATACGACTCACTATAGGGTTCACGAAGGAAGAGCTGG TAATACGACTCACTATAGGACGAAATCCTGACTGTTCGG
A2MRAP	qPCR	CGCGTTCCGTGGGTTGGACA CCGGCGAACGTTTTGCTCGC
LRTP	dsRNA	TAATACGACTCACTATAGGGCTCGATCTTGACGAGAACC TAATACGACTCACTATAGGGTACAGCTCATCCAACCCGT
LRTP	qPCR	CCGGCGAACGTTTTGCTCGC TCCGATCGAGCCGATCAAAG
SRPN10	dsRNA	TAATACGACTCACTATAGGTGGTCATTTCCCCGTTCTCG TAATACGACTCACTATAGGTCACTCTCGGCGAAGTTGAC
SRPN10	qPCR	TCGGACAGTCGAACAGCTTC CGAGAACGGGGAAATGACCA
R2RSP1	dsRNA	TAATACGACTCACTATAGGGCCGGAAAATGCAACGCTTA TAATACGACTCACTATAGGTGATAGTATGACACGCGGGC
R2RSP1	qPCR	AACGACATCGCACTGGTGAA TCGGAGTATGCGATGGGTTG
R2RSP2	dsRNA	TAATACGACTCACTATAGGGTCGGTAGCAGTTCCGTTGA TAATACGACTCACTATAGGCACTCGTGAGCACTTCCTGT
R2RSP2	qPCR	GCGGATGAGGACGATCAGTT TCGGAGTATGCGATGGGTTG
SEPR1	dsRNA	TAATACGACTCACTATAGGTACCGTACCAGATTGCGCTC TAATACGACTCACTATAGGCGGTTGGATGTACTGGCTGT
SEPR1	qPCR	ACAGCCAGTACATCCAACCG GCGTCCGTATCCCGAAATCA
TRYPP	dsRNA	TAATACGACTCACTATAGGCCTACGTCAACCGAGTCGTC TAATACGACTCACTATAGGCCTACTAGACAGTGAGCGGC

TRYPP	qPCR	CTAGGGTGCCTCGGCTAGTT TTCTTCGCCACTTCTCCACC
ACEP	dsRNA	TAATACGACTCACTATAGGGAGAGCGGGAGATTAGTGGC TAATACGACTCACTATAGGCGTCGGCAAAGTAGCGATTG
ACEP	qPCR	CTACAGCAACAGCTGACCGA CGTCGGCAAAGTAGCGATTG
SEPR2	dsRNA	TAATACGACTCACTATAGGTGAATGTCTCCGCCAGCTTT TAATACGACTCACTATAGGGAACCGTCTTGACGCGATTG
SEPR2	qPCR	CTACACCGTTGGGAGCAAGT AACCCACTCGCTGAAGTAGC

Table 2.1: PCR primers used in this study. "Gene name" displays the name of the gene targeted by the primer, "Primer type" indicates whether this primer was used for the creation of dsRNA or for quantitative real-time PCR and "Primer sequences" gives the sequences of both the forward (top) and reverse (bottom) primers.

Dr. Jake Tu (Virginia Polytechnic Institute and State University) using Mascot (Matrix Science) and Proteome Discoverer (v1.2 Thermo Scientific) with the high peptide confidence filter. The resulting data were considered significant if the ratio of GM:WT protein levels was >0.75 on a $\log_2$ scale. Sequences obtained from the mass spectrometer were compared to previously annotated *A. gambiae* gene sequences using blastp (Altschul et al. 1990) in order to assign them putative functions and GO terms.

dsRNA-mediated gene silencing

Double-stranded RNAs (dsRNAs) targeting selected genes were synthesized from PCR products using the HiScribe T7 *in vitro* transcription kit (NEB). Adult female mosquitoes (3-4 days old) were anesthetized on ice and injected with 69 nl of 3 µg/µl dsRNA targeting a gene of interest or GFP as a control, then maintained under normal mosquito rearing conditions. At 3 days post-injection, groups of 10 mosquitoes were collected for silencing efficiency measurement using qRT-PCR. Primers used for the PCR amplification of oligos were designed using the Primer3 program

(http://frodo.wi.mit.edu) and are listed in table 2.1. Silencing of the genes was verified by qRT-PCR 3 days post-injection (Figure 2.2).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA used to verify gene knockdown was extracted from whole mosquitoes with their heads and legs removed, while total RNA used to verify the microarray results was collected from dissected midguts or fat bodies using a Qiagen RNEasy kit according to the manufacturer's instructions. qRT-PCR was carried out using Sybr Green PCR Master Mix (ABI) on an ABI StepOnePlus Real-Time PCR System with the ABI StepOne Software. Transcript abundance was normalized to mosquito ribosomal protein S7 gene levels and the -fold change of each gene was calculated using the ΔΔct method. Primers were designed using the Primer3 program (http://frodo.wi.mit.edu) and are listed in table 2.1.

P. falciparum *infections*

P. falciparum challenges were performed according to a standard laboratory protocol: Three days after dsRNA injection, mosquitoes were fed on human blood containing NF54W strain *P. falciparum* gametocytes through a membrane feeder at 37° C. Unfed mosquitoes were discarded and mosquitoes were maintained as usual for 7 days, at which point their midguts were dissected and stained with 0.1% mercurochrome for oocyst enumeration by manual counting under a light microscope (Olympus).

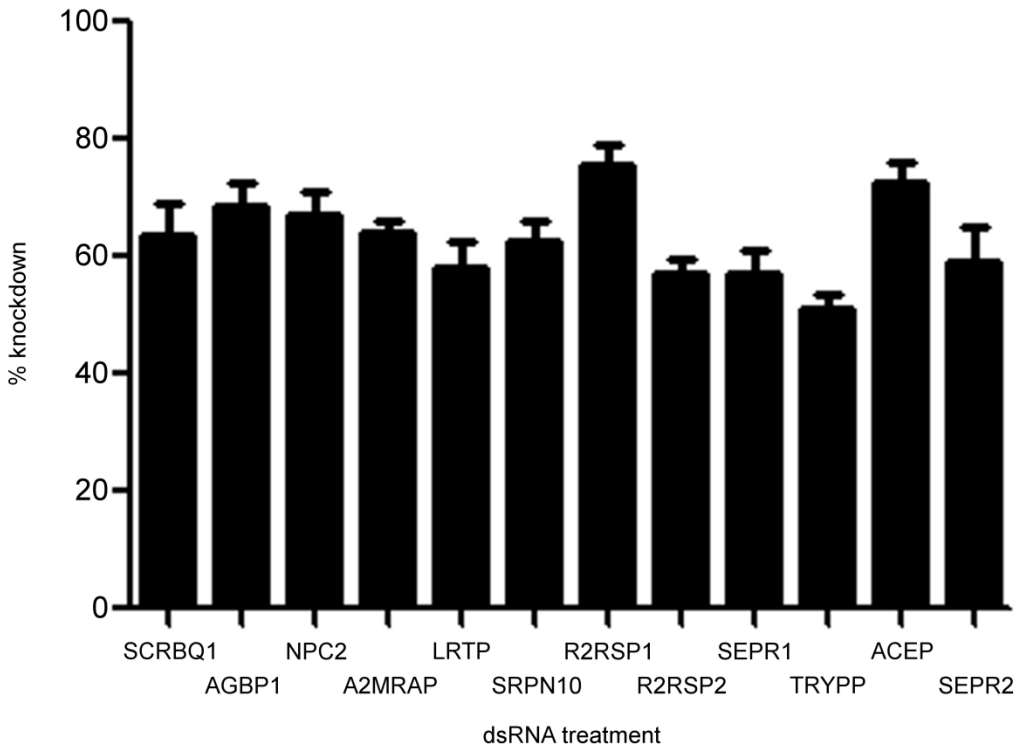

Figure 2.2: qRT-PCR verification of dsRNA mediated gene knockdown. mRNA was collected from dsRNA injected mosquitoes and used for qRT-PCR to measure the percent knockdown of the gene. All genes showed greater than 50% knockdown.

Enumeration of midgut bacteria

The number of colony forming units (CFUs) of midgut bacteria for gene-silenced sugar or blood-fed mosquitoes was counted as previously described: Mosquitoes were surface-sterilized with ethanol and rinsed in PBS before their midguts were dissected into PBS. Midguts were then homogenized in PBS, and serial dilutions were plated onto LB agar plates and incubated at room temperature. Three days after plating, the number of colonies per plate was counted, and the total number of culturable bacteria per midgut was calculated. Samples were collected for sugar-fed mosquitoes 3 days after gene

silencing, while other mosquitoes were provided a blood meal 3 days after gene silencing, with dissections being performed 24 h later.

Statistical Analyses

Statistical analyses were performed using the GraphPad Prism 5 software (Graphpad Software). The tests used are indicated in the Results section and figure captions.

2.3 Results and Discussion

2.3.1 Expression of active Rel2 leads to broad transcriptome and proteome changes

Using whole-genome microarrays, we compared the mRNA abundance of all predicted *An. stephensi* genes in transgenic Rel2-overexpressing (Dong et al. 2011) and WT mosquito lines at two time points following a blood meal in order to identify the Rel2-driven changes in the midgut and fat body transcriptomes. Mosquitoes with Rel2 expression driven by the midgut-specific carboxypeptidase promoter (CP line) were assayed at 6 and 12 h post-blood meal (PBM)**),** and samples with Rel2 expression driven by the fat body-specific vitellogenin promoter (VG line) at 12 and 18 h PBM. Selection of these time points was based on the recombinant Rel2 induction profile in the two tissues (Dong et al. 2011). At 6 h PBM, there were 190 up-regulated and 94 down-regulated genes in the midguts of CP line mosquitoes (Figure 2.3A), and these totals increased to 645 up-regulated and 596 down-regulated genes at 12 h (Figure 2.3A). In VG line mosquitoes, there were 173 up-regulated and 152 down-regulated genes in the fat body at 12 h PBM and 203 up-regulated and 189 down-regulated genes at 18 h (Figure 2.3A). This is similar to the number of differentially regulated genes seen in the

fat bodies of Rel2 overexpressing *Ae. aegypti* mosquitoes, which upregulated 123 and downregulated 176 downregulated genes following Rel2 activation (Zou et al. 2011). A total of 71 transcripts were regulated by recombinant Rel2 activation in both the CP midgut and VG fat body, with 19 being regulated in the same direction at 12 h PBM (10 up-regulated in both tissues and 9 down-regulated in both tissues) and 20 being up-regulated in the midgut and down-regulated in the fat body; 32 displayed the opposite mRNA abundance pattern (Figure 2.3B). It is unlikely that a transcription factor would up-regulate and down-regulate the same gene in different tissues, so it is much more likely that the differences in expression pattern are related to interactions with other transcription factors, feedback loops, or mRNA or protein degradation rates. Because we were looking at global changes following Rel2 induction, we could not differentiate between an mRNA directly up-regulated by Rel2 and another that was down-regulated because of a silencing factor, or an mRNA with a long half-life compared to another that was degraded by a micro-RNA that was up-regulated by Rel2.

Predicted gene ontology (GO) categories were assigned to genes based on homology to previously annotated *An. gambiae* genes (vectorbase.org). In both tissues and at all time points, the predicted GO category with the greatest number of significantly regulated genes was the diverse functional category (a total of 508 differentially regulated genes at 12 h PBM) followed by the unknown category (328 total differentially regulated genes at 12 hours PBM). This is to be expected, since these two GO categories represent over half of the annotated genes in the *An. gambiae* genome (vectorbase.org), and therefore the predicted GO terms for our *An. stephensi* genes. The broad functional spectrum of genes assigned to the diverse category reflects the far-reaching effects of Rel2 activation.

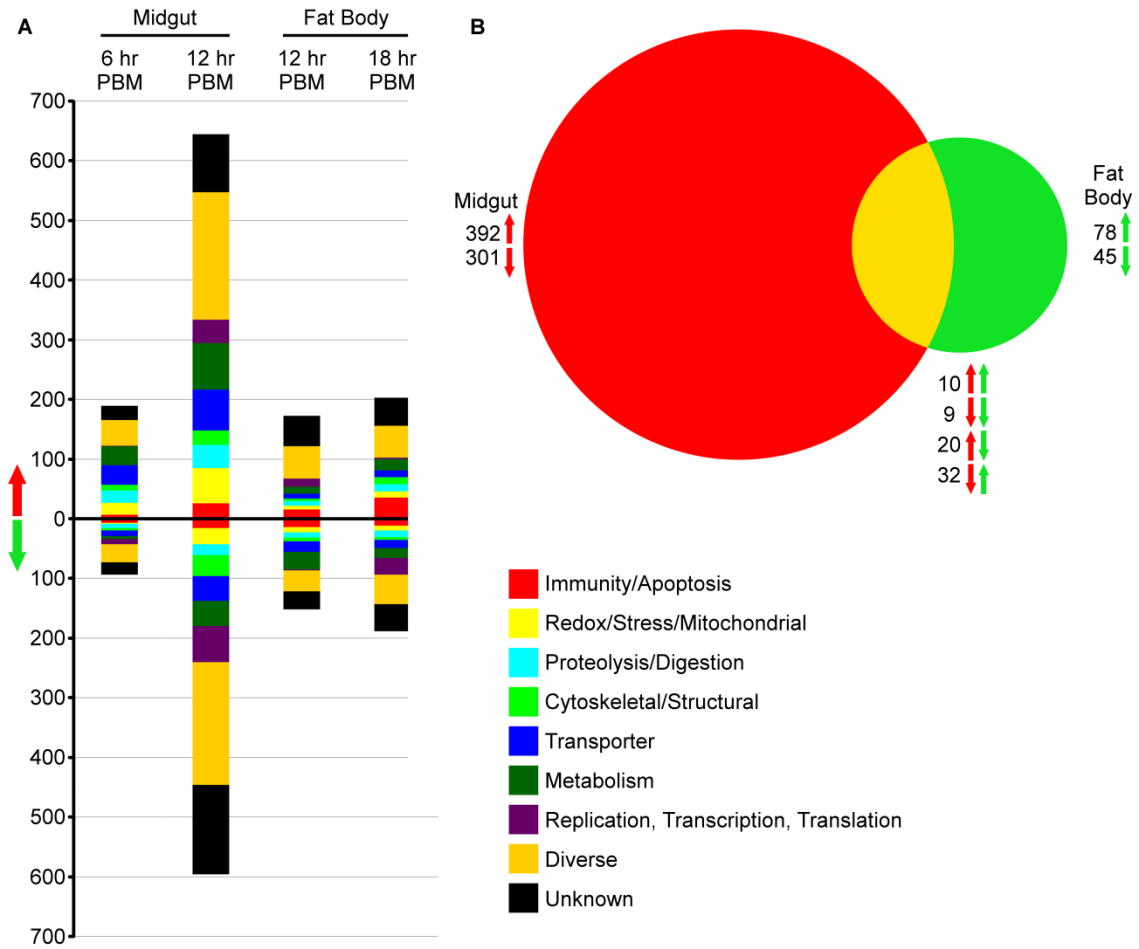

Figure 2.3: Global changes in transcript levels in transgenic *A. stephensi* following Rel2 induction. A) The total number of genes significantly up- or down-regulated that are predicted to be in each GO category. Genes were considered significantly differentially regulated if the -fold change was>= 0.75 on a $\log_2$ scale. B) Venn diagram comparing the total number of regulated transcripts between the midgut of CP line mosquitoes and fat body of VG line mosquitoes at 12 h PBM. Red arrows correspond to midgut samples, and green arrows correspond to fat body samples; the arrow direction indicates significant up- or down-regulation.

Because Rel2 is the major transcription factor of the IMD pathway, we conjectured that numerous genes assigned to the immune/apoptosis GO category would be up-regulated following Rel2 expression. There were 26 genes from this category that were up-regulated in the midgut of CP mosquitoes at 12 h PBM, representing 4% of the total up-regulated genes at that time point. There were also 16 down-regulated genes in this

category at the same time. In the VG line fat body, there were 36 up-regulated immune genes at 18 h PBM, representing 17.7% of up-regulated fat body genes at 18 h PBM, with 12 down-regulated at the same time. Highly up-regulated immune genes included both known anti-*Plasmodium* effectors, such as TEP1 and LRIM1 (Blandin et al. 2004, Povelones et al. 2009), and genes that have not yet been associated with *Plasmodium* resistance but that have shared domains with known anti-*Plasmodium* effectors, such as multiple leucine-rich repeat and fibrinogen domain-encoding genes and two MD-like genes (Riehle et al. 2008, Garver et al. 2009, Povelones et al. 2009). While a smaller proportion of immune genes were found to be differentially regulated in the *An. stephensi* genome following Rel2 expression than in *Ae. aegypti* (Zou et al. 2011), many of the same types of immune genes are represented in the upregulated group, including thio-ester proteins and proteins containing leucine rich repeats. Representatives from other GO categories, such as serine proteases in the Proteolysis/Digestion group, redox responsive genes in the Redox/Stress/Mitochondrial category, and many others, are likely to be relevant for reciprocal interactions between *Plasmodium* and the mosquito. Other up-regulated genes such as cytochrome P450s may play a role in the mosquito's resistance to insecticides (David et al. 2013) and thereby affect the ability of these transgenic mosquitoes to survive and compete in the wild.

To determine changes in midgut and fat body proteomes following Rel2 activation in these tissues, we used iTRAQ to quantify the relative amounts of all proteins in Rel2-overexpressing transgenic mosquitoes relative to their wild-type conspecifics both before and 24 h after a blood meal. Filtering for only high confidence peptides, we identified 31,392 peptide spectrum matches (PSMs) corresponding to 8,574 peptides that

mapped to 2,244 unique protein contigs previously annotated by the Jake Tu Lab (Virginia Polytechnic Institute and State University). These proteins were assigned putative names and functions through a BLASTp search against the *An. gambiae* genome, yielding 2041 unique proteins, 2024 of which had significant similarity (BLASTp e-value < 0.01) to *An. gambiae* genes. Prior to a blood meal, the transgenic mosquito midgut displayed significantly higher levels of 89 proteins and lower levels of 45 proteins. The transgenic mosquito fat body displayed higher levels of 204 proteins and lower levels of 31 proteins prior to the blood meal (Figure 2.4A). One reason for the differential expression of these proteins in the absence of Rel2 induction may be that they may represent genes with permanently altered expression patterns, either as a result of position effects of transgenesis or adaptation during the numerous generations since the insertion of the transgene. Alternatively, there may be leaky expression from the CP and VG promoters, as has been observed before for the VG promoter for some autogenous mosquito species (Provost-Javier et al. 2010), though not in *Anopheles* spp. mosquitoes. The reason for this leakiness could be further explained by examining the expression profiles of multiple lines with different transgene insertion locations. However, due to the effort required to maintain these lines, only one line of each strain, with the most potent anti-*Plasmodium* activity, has been kept and therefore we are limited to observing the changes in a single line for each tissue. As expected, at 24 h PBM, there were many more proteins displaying significantly altered abundances. Specifically, after a blood meal there were 1,230 up-regulated and 64 down-regulated proteins in the midgut and 26 up-regulated and 185 down-regulated proteins in the fat body (Figure 2.4A). Of these, only 22 were significantly regulated in the same direction (10 up and 12 down) in both

the midgut and the fat body, while 78 were significantly regulated in opposite directions (77 up in the midgut and down in the fat body, and 1 with the reverse) (Figure 2.4B). As with transcripts, the GO category with the greatest number of differentially regulated proteins assigned to it was the diverse category (493 total differentially regulated proteins at 24 h PBM), although all the GO categories were represented, especially in the list of proteins up-regulated in the midgut at 24 h PBM. In the midgut, there were 50 proteins, or 4% of the total up-regulated proteins, belonging to the immune GO category that were significantly up-regulated at 24 h PBM, but there were only 2 significantly down-regulated immune proteins, or 3.1%, at that time. In the fat body at 24 h PBM, there were only 2 significantly up-regulated immune proteins, but these proteins represent 7.7% of the total significantly up-regulated proteins, and there were 7 significantly down-regulated proteins representing 3.7%. As for the transcriptome, there was a functionally diverse set of up-regulated immune proteins, including some corresponding to genes found to be regulated in the microarray–based transcriptome analyses, such as *An. gambiae* MD2-like protein 6 (AgMDL6) and neuronal leucine-rich repeat protein 3 (NLRR3). Other significantly up-regulated proteins were not found to be significantly up-regulated in the same tissue at the transcript level, but the regulated transcriptome and proteome sets contained many genes belonging to the same families, such as leucine-rich repeat and fibrinogen domain-containing proteins. Interestingly, the levels of both Toll and Rel1, which are Toll pathway-associated genes, were significantly up-regulated at the protein level following Rel2 activation, possibly indicating an interplay between the IMD and Toll pathways.

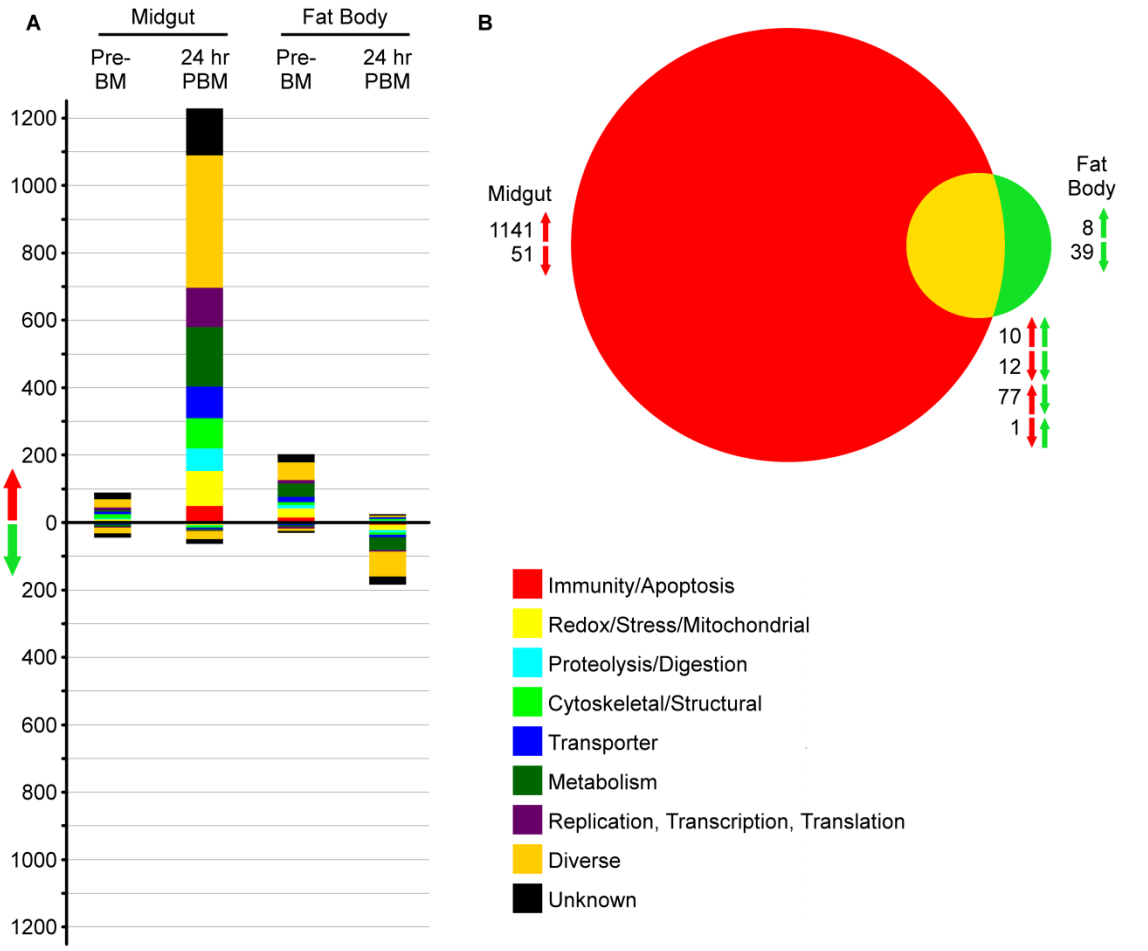

Figure 2.4: Global changes in protein levels in transgenic *A. stephensi* following Rel2 induction. A) The total number of proteins significantly up- or down-regulated that are predicted to be in each GO category. Genes were considered significantly differentially regulated if the ratio of transgenic to wild type was>= 0.75 on a $\log_2$ scale. B) Venn diagram comparing the total number of regulated proteins between the midgut of CP line mosquitoes and fat body of VG line mosquitoes at 24 h PBM. Red arrows correspond to midgut samples, and green arrows correspond to fat body samples; the arrow direction indicates significant up- or down-regulation.

In both the transcriptomic and proteomic analyses, we observed differential expression of genes belonging to various functional classes (Figures 2.3 and 2.4). Here, we present a brief overview of the several classes of genes that we believe are relevant to the Rel2 pathway or provide interesting insights on the physiological influence of this pathway.

Immune-related genes: In total, 102 separate immune-related genes were up-regulated under at least one experimental condition, while 44 were down-regulated. These genes represent a large number of different families of immune genes, including 8 leucine-rich repeat containing proteins, 5 tep proteins, 14 CLIP domain serine proteases, and 7 serine protease inhibitors, as well as many anti-microbial peptides and other genes. Interestingly, in addition to IMD pathway-associated genes such as Rel2, a number of Toll pathway-associated genes, including Toll and Rel1, were also significantly up-regulated, indicating that there may be interaction between the two major immune pathways. Similarly, Hop and JNK, components of the Jak/Stat and JNK pathways, were also up-regulated. These data indicate that the various immune pathways do not act in isolation and may instead act together to attain a broader immune response. The JNK pathway has recently been shown to play a role in *Plasmodium* resistance (Garver et al. 2013), and interaction between the JNK and IMD pathways may lead to greater *Plasmodium* immunity. Up-regulation of AMP genes such as defensin and cecropins likely plays a direct role in the control of bacteria and other pathogens (Meister et al. 2005), and similar upregulation of AMP expression has been seen following Rel2 induction in *Ae. aegypti* (Zou et al. 2011), while other genes such as the serine proteases likely trigger cascades to amplify and diversify the response (An et al. 2010).

Digestive: 485 digestion-related genes were up-regulated across the experimental conditions, and 280 were down-regulated. Among these were genes involved in both protein and sugar digestion, as well as many Ras family proteins. Numerous proteolysis related genes were also found to be differentially regulated in the *Ae. aegypti* Rel2 regulated transcriptome (Zou et al. 2011). Up-regulated protein digestion-related genes,

such as 26s proteosome subunits and serine/threonine phosphatases, work as part of the system for digesting blood meals following ingestion and may aid in the control of bacterial proliferation in the midgut following a blood meal. Ras family proteins are GTPases involved in cell proliferation and signaling, some of which have been implicated in *Drosophila* immunity (Ye and Zhang 2013). While evidence from *Drosophila* implicates the Ran subfamily of Ras genes in the phagocytosis of virus-infected cells, other Ras genes may play a role in phagocytosis of other infectious organisms. Alternatively, GTPases, such as Ras family proteins, and kinases may act in a similar fashion to serine proteases and related genes to amplify and diversify the signal from the IMD pathway.

Cell structure genes: There were also 82 up-regulated and 48 down-regulated genes in the Cell Structure GO category. These included genes vital for muscle function or cell motility, such as 7 up-regulated and 3 down-regulated actins and 13 up-regulated and 8 down-regulated myosins; genes used for cell division, such as 3 up-regulated tubulins; and numerous cuticular proteins, actin- and chitin-binding proteins, and various other proteins. Previous research has shown that the anti-*Plasmodium* immune response involves remodeling of the midgut epithelium (Han and Barillas-Mury 2002), as does bacterial resistance in the *Drosophila* midgut (Buchon et al. 2013). In *Drosophila*, infected cells are expelled into the midgut lumen and must be regenerated, and both processes require action by the cell structure and motility system. Other studies have also shown that remodeling of the cytoskeleton is necessary for successful *Plasmodium* infection and traversal of the midgut (Han and Barillas-Mury 2002), so changes in the cytoskeleton by the immune system may be important for resistance to infection.

Redox genes: Finally, many redox and stress-related genes showed differential regulation. Components of the ROS system have been implicated in resistance to both bacteria (Oliveira et al. 2011) and *Plasmodium*, and multiple NOS and mitochondrial carrier (Goncalves et al. 2012) genes are up-regulated following IMD pathway induction. The up-regulation of ROS-related genes by the IMD pathway may be a link between the various ways of fighting off midgut infection by both *Plasmodium* and bacteria. By up-regulating these genes, the mosquito can attack pathogens with both ROS and AMPs at the same time, increasing the potential for clearance. Many components of the oxidative phosphorylation system, including various reductases and oxidases, were also up-regulated under at least one condition, indicating that the mosquito may increase ATP production in order to compensate for energy use by the immune system.

Genes with few representatives: In addition to the gene families with large numbers of representatives listed above, there were also gene families with very few differentially regulated representatives. For instance, odorant receptors and other sensory proteins were almost completely absent from the differentially regulated datasets, despite having many representatives in the genome (vectorbase.org). Thus, the mosquito IMD pathway likely does not greatly affect the ability of mosquitoes to sense their environment.

While we have identified a large number of transcripts and proteins that displayed altered expression in our genetically modified mosquitoes following up-regulation of Rel2, not all of these genes are likely to be controlled directly by Rel2, and there are many Rel2-regulated genes that were not discovered through our approach. We tested only a limited number of time points, and there may be short-lived transcripts and

proteins that were degraded before we collected our samples. Similarly, some genes may take longer to transcribe and translate than others, and our time points may have been too early to observe the changes in expression. Other genes may require the binding of different transcription factors not present in this study in addition to Rel2, and thus they would not be differentially regulated following the up-regulation of Rel2 alone. Conversely, some genes having promoter sequences with low affinity for Rel2 may be differentially regulated in our transgenic mosquitoes, likely because of the overabundance of an active Rel2 form in the system. The differential regulation of many genes upon activation of recombinant Rel2 is also likely to represent a secondary effect and a general physiological response to immune activation. However, we believe that the time points we have chosen are well chosen to capture both the timing of Rel2 up-regulation in our mosquitoes and the physiologically relevant times for *P. falciparum* invasion of the midgut, in the under-studied mosquito vector *An. stephensi*.

2.3.2 Correlation between mosquito transcript and protein expression levels

Because we measured both transcript abundance and protein abundance, we were able to compare the expression of genes at the two levels in order to assess a correlation between transcript levels and protein abundance. Previous studies in other organisms have shown only weak concordance between transcripts and proteins (de Sousa Abreu et al. 2009, Vogel and Marcotte 2012), but such studies are usually restricted to unicellular organisms or cell lines, and this study provided an opportunity to expand this knowledge to multicellular eukaryotes.

When we looked at all the genes that displayed a significant difference at the protein abundance level at 24 h PBM and a significant difference at the transcript abundance level at one of the two time points, we saw no significant correlation between protein and transcript levels in the midgut when we used a linear model (Figure 2.5A). However, we saw a significant correlation for the fat body (Figure 2.5B). Our assays generated data for 1,273 genes at both the transcript and protein levels in the midgut, 240 of which were significantly regulated in the same direction at both the protein and transcript levels, while 119 were significantly regulated in opposite directions. In the fat body, we obtained data for 1,538 genes at both the transcript and protein levels, with 33 significantly being regulated in the same direction at both protein and transcript levels and only 8 significantly regulated in opposite directions. The lack of significant correlation between mRNA and protein levels in the midgut is likely due to the wide variety of genes that are differentially expressed in that tissue and the various post-transcriptional modifications and regulatory mechanisms involved. The lack of correlation is also indicative of the large proportion of genes that are not directly regulated by the Rel2 transcription factor. Furthermore, factors such as transcript and protein degradation rates, miRNAs, mRNA secondary structure, the presence of other transcription regulators, and the availability of ribosomes and amino acids to build proteins could all contribute to differences between the abundance of mRNA and proteins (Maier et al. 2009, Vogel and Marcotte 2012). For instance, if the mRNA of a particular transcript is quickly degraded but the protein is long-lived, or vice-versa, then an increase in the transcription of that gene will not necessarily lead to a measurable change in the gene expression at both levels. Similarly, other transcriptional and translational

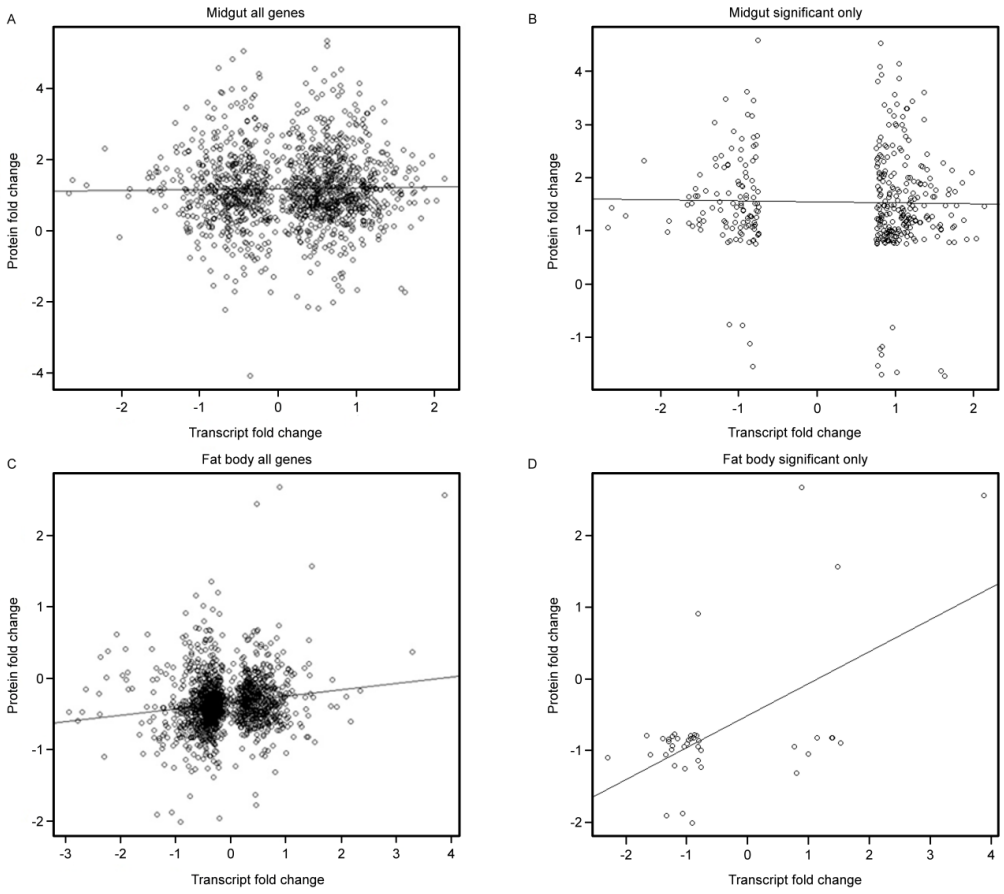

Figure 2.5: Correlation between mRNA and protein levels in the mosquito midgut (A,B) and fat body (C,D) following a blood meal. In the midgut there was no significant correlation between mRNA and protein levels whether looking at all genes (A, r^2=0.0003, F-statistic = 0.4276, p-value = 0.5133) or only significantly regulated genes (B, r^2=.0005, F-statistic = 0.1766, p-value = 0.6746), while in the fat body there was a significantly correlation both when looking at all genes (C, r^2=0.02183, F-statistic = 34.28, p-value < 0.001) and when looking at only significantly regulated genes (D, r^2=0.3213, F-statistic = 18.47, p-value < 0.001).

regulators, including transcription factors, miRNAs, and feedback loops, may promote or inhibit transcription and translation differentially, leading to discordance between mRNA and protein levels. Finally, it is possible that the time points at which we measured expression levels did not adequately capture the timing of gene expression. Our previous work indicates that Rel2 expression levels should be high at 12 h and remain elevated for many hours afterward in both the midgut and fat body, and protein levels would, if

directly governed by transcription, follow soon after. An increase in transcripts, even if not immediately translated, could allow mosquitoes to increase the abundance of a protein quickly if other signals necessary for the translation of that gene were present. Similarly, differential degradation of transcripts and proteins may allow mosquitoes to remove unnecessary gene products, even if transcription is initiated by a promiscuous promoter. Other studies have also shown a lack of correlation between mRNA and protein levels (You and Yin 2000, Gedeon and Bokes 2012). Our results show that a lack of correlation between mRNA and protein expression levels is also prominent in mosquitoes, likely providing mosquitoes a fine level of control over the proteins expressed in their cells.

2.3.3 Identification of novel anti-Plasmodium immune genes

The analyses of transcript and protein abundance following Rel2 induction in transgenic mosquitoes allowed us to select a variety of genes to investigate further for involvement in the mosquito's immune defense. Because Rel2 is an IMD pathway-associated transcription factor, we expected a large number of significantly up-regulated immune genes. Similarly, because this immune pathway is responsible for the mosquito's resistance to *Plasmodium* infection (Garver et al. 2009), we were interested in identifying IMD pathway-regulated genes that control resistance to *P. falciparum*. We began by selecting five of the immune genes with the greatest up-regulation at the protein level at 24 h PBM in the midgut. We chose to consider only the protein level for these genes because it takes approximately 24 h for *P. falciparum* to exit the midgut lumen, so proteins with increased expression at this time will be available to act against the parasite.

From the 20 most abundant immune proteins, we chose 5 that may have anti-*Plasmodium* activity for further testing based on the level of up-regulation and predicted functions based on sequence homology. Specifically, we chose a class B scavenger receptor containing a SCRBQ1 domain (SCRBQ1), bacteria response protein 1 (AGBP1), Neimann-Pick type C-2 (NPC2), alpha-2-macroglobulin receptor-associated protein (A2MRAP), and a leucine-rich transmembrane protein (LRTP). The selected genes were knocked down by RNAi prior to parasite exposure, and the resulting impact on *P. falciparum* infection of the mosquito midgut was assayed by oocyst counting (Figure 2.6). RNAi-based depletion of SCRBQ1, AGBP1, and NPC2 had no significant effect on *Plasmodium* infection levels; however, depletion of A2MRAP and LRTP led to significant increases in the number of oocysts per midgut (Kruskal-Wallis test, $p<0.0001$; Dunn's post-hoc test: A2MRAP, $p<0.01$; LRTP, $p<0.001$). While the median number of oocysts was decreased following both A2MRAP and LRTP knockdown, none of the dsRNA treatments had a significant effect on oocyst prevalence, even though previous studies have shown that Rel2 knockdown by RNAi leads to an increase in oocyst prevalence. This difference in result may stem from the fact that the genes we knocked down are only a part of the whole immune response to *Plasmodium* infection, and their knockdown may not be efficient enough to affect the prevalence, whereas the full complement of IMD pathway-regulated immune effectors together cause a greater effect.

SCRBQ1 (ASTE009112/AGAP010132) is homologous to the *Croquemort* (CRQ) gene in *D. melanogaster*, which is essential for efficient phagocytosis of apoptotic cells in *Drosophila* embryos (Franc et al. 1996, Franc et al. 1999), and may play a role in anti-*Plasmodium* defense through apoptosis or phagocytosis (Hurd et al. 2006, Blandin and

Levashina 2007). AGBP1 (STE009712/AGAP008061) is one of a number of bacterial infection-responsive proteins that have been identified in *An. gambiae,* and is up-regulated following infection with *P. berghei* (Dong et al. 2006b). However, *P. berghei* infection is principally controlled by the Toll pathway (Garver et al. 2009), so the relevance of this protein in defending against *P. falciparum* infection may be minimal. NPC2 (ASTE004995/AGAP002851) is a small, highly conserved, secreted protein that plays an important role in regulating sterol homeostasis in *Drosophila* (Ioannou 2007). *Plasmodium* parasites are unable to synthesize their own sterols and must scavenge these molecules from their host (Bano et al. 2007),so changes in the abundance of sterols may affect the ability of *Plasmodium* to infect mosquitoes. NPC2 also plays an important role in dengue resistance of *Ae. aegypti* mosquitoes and may play a role in a variety of infection systems (Jupatanakul et al. 2013). A2MRAP (ASTE011001/AGAP003521) is a protein associated with the receptor for alpha-2-macroglobulins. Alpha-2-macroglobulin is an abundant protein that binds to a variety of ligands and is involved with the lectin-dependant cytolytic pathway in arthropods (Armstrong and Quigley 1999). The diversity of ligands to which alpha-2-macroglobulin can bind and its importance for the lysis of cells indicate that it, and its receptors, could be important for the lysis of *Plasmodium* infected cells and help mosquitoes fight off the parasite. Finally, LRTP (ASTE008359/AGAP007061) bears structural similarity to other proteins containing leucine-rich repeats. Two such proteins, LRIM1 and APL1C, have been shown to be important for the anti-*Plasmodium* defense in *An. gambiae* (Dong et al. 2006b, Povelones et al. 2011), suggesting that similar proteins may also act against *Plasmodium* in *An. stephensi*.

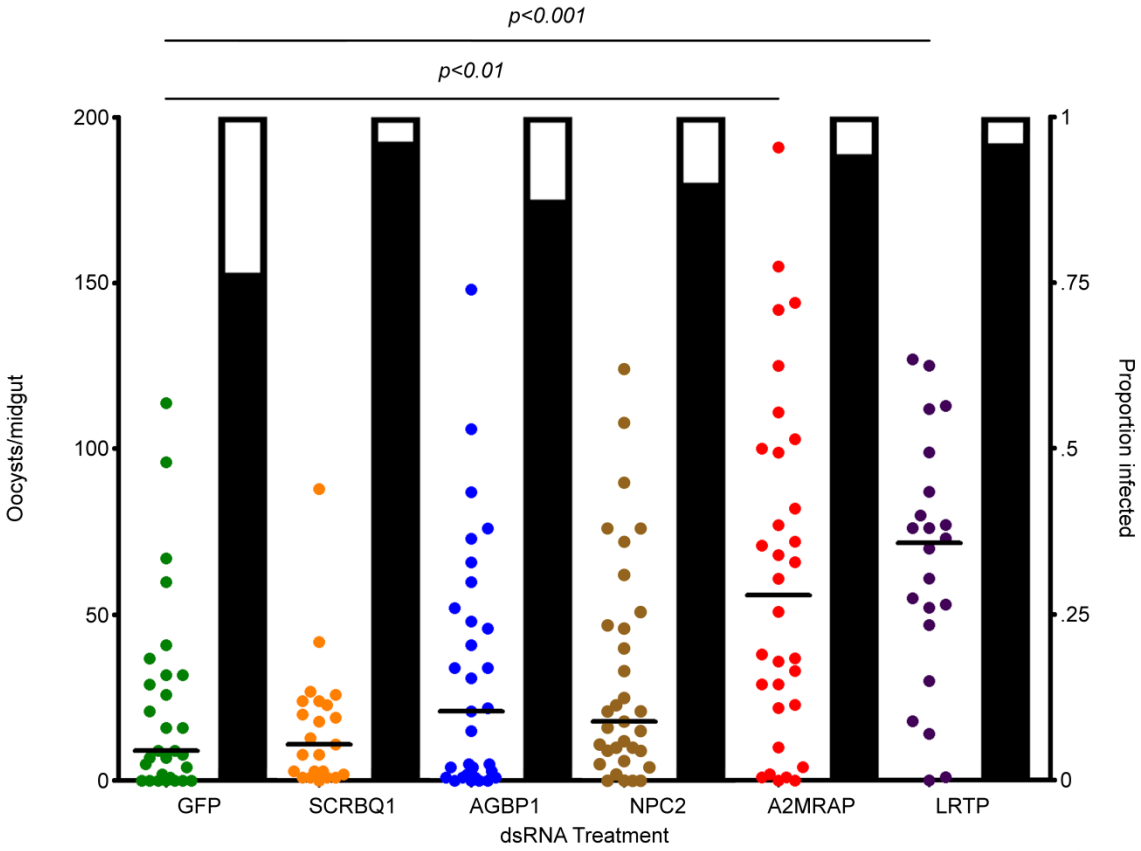

Figure 2.6: *P. falciparum* infection intensity following RNAi knockdown of immune genes. The number of oocysts per midgut of wild-type *A. stephensi* following RNAi-mediated depletion of GFP, SCRBQ1, bacterial response protein (AGBP1), Niemann-Pick type-C (NPC2), alpha-2-macroglobulin (A2MRAP), or leucine-rich transmembrane protein (LRTP). Depletion of both A2MRAP and LRTP led to a significant increase in the number of oocysts per mosquito midgut. Each circle represents a single midgut, and horizontal black bars represent the median of the sample. Significance was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test to compare immune-depleted mosquitoes to GFP controls. Significance was assessed at α=0.05. Supplementary data for this figure is given in table 2.2.

The lack of an increase in *Plasmodium* oocysts per midgut following knockdown of SCRBQ1 is likely a result of gene redundancy, with alternative proteins also controlling apoptosis CRQ is largely expressed in the *Drosophila* embryo and not in adults, and it is possible that SCRBQ1 is similarly more active during mosquitoes' immature stages than in adults. Both AGBP1 and NPC2 are known to be immune

modulators for other parasites and may simply display specificity for bacteria and viruses and not act against *Plasmodium*. Knockdown of both A2MRAP and LRTP led to significant increases in *Plasmodium* infection, indicating that these proteins are important in modulating *P. falciparum* infection in the mosquito.

*2.3.4 Serine proteases affect mosquito anti-*Plasmodium *defenses*

A large number of proteases and digestive enzymes were highly up-regulated at both the protein and transcript levels. Serine proteases and other proteolytic enzymes are often part of proteolytic cascades that can lead to the amplification of signals that control downstream effector mechanisms (An et al. 2010). In mosquitoes, some serine proteases have been implicated in blood digestion (Yang and Davies 1971), the anti-*Plasmodium* defense (Volz et al. 2005, Blumberg et al. 2013), signal transduction, and many other diverse functions. The TOLL immune pathway, for instance, is activated through a serine protease-dependent signaling cascade (Ligoxygakis et al. 2002, Weber et al. 2003). A number of serpins are involved in the prophenoloxidase (PPO) activation cascade, an important part of the innate immune system (Christophides et al. 2002, Ligoxygakis et al. 2003, Weber et al. 2003), but the functions of many other serpins have yet to be elucidated. The presence of many proteolytic regulators, including serine proteases and their inhibitors, in the highly up-regulated gene group at both the transcript and protein levels indicates that serine proteases play a role in the IMD pathway-based immune response and the mosquito anti-*Plasmodium* defense. It may also help to explain the broad diversity of genes affected by increased expression of Rel2, since serine protease-

dependent signaling cascades can both amplify and diversify the signal, causing changes in the regulation of many different genes.

Given the significant up-regulation of numerous proteases and related enzymes at both the protein and transcript levels, we decided to investigate a number of them further. Serine protease inhibitor 10 (SRPN10), Rel2-responsive serine protease 1 (R2RSP1), Rel2-responsive serine protease 2 (R2RSP2), serine protease precursor 1 (SEPRP1), trypsin precursor (TRYPP), angiotensin converting enzyme precursor (ACEP), and serine protease precursor 2 (SEPRP2) were all chosen because they showed at least a 2-fold induction at the transcript level at either 6 or 12 h PBM and at the protein level 24 h PBM in the midgut. Silencing of R2RSP2, ACEP, and SEPRP2 significantly increased oocyst loads, suggesting that these factors are *P. falciparum* antagonists (Figure 2.7) (Kruskal-Wallis test, $p<0.0001$; Dunn's post-hoc test for R2RSP2, $p<0.01$, ACEP $p<0.05$, SEPRP2 $p<0.001$). However, as with the immune genes, none of the silenced genes had a significant effect on oocyst prevalence. Again, this may be due to the limited activity of only a few genes, when compared to the overall effects of the full IMD pathway-based response. Serine proteases act in diverse processes in mosquitoes and other dipterans, including activating signaling cascades (such as the Toll and PPO cascades) and regulating development. SRPN10 (ASTE007248/AGAP005246) is one of many serine protease inhibitors (serpins) found in mosquitoes. Studies in *An. gambiae* have shown that some isoforms of SRPN10 are up-regulated during the parasite's traversal of the midgut and may be involved in anti-*Plasmodium* defense (Danielli et al. 2005). R2RSP1 (ASTE006240/AGAP007142) and R2RSP2 (ASTE014104/AGAP007165) are both examples of trypsin-like serine proteases. SEPRP1 (ASTE009202/AGAP005065) and

SEPRP2 (ASTE010540/AGAP005310) are both precursors to serine proteases and may be involved in many of the same functions as R2RSP1 and R2RSP2. TRYPP (ASTE010330/AGAP006709) is a precursor for a chymotrypsin which are proteolytic enzymes and form a subset of serine proteases that play a role in the digestion of mosquito blood meals (Yang and Davies 1971). Serine proteases similar to those identified in this study cleave a variety of targets and may have many different functions, including the regulation of anti-*Plasmodium* activity. Finally, ACEP (ASTE004060/AGAP004563) is a precursor for angiotensin converting enzyme (ACE), a family of proteins found in the hemolymph of insects and cleave a broad range of substrates (Riordan 2003, Burnham et al. 2005). While various ACE-like proteins have been studied in *An. gambiae* and an immune function has been suggested, their potential effect on *Plasmodium* has not been investigated.

Two serpins, SRPN6 and SRPN7, have previously been shown to play roles in the anti-*Plasmodium* defense (Abraham et al. 2005, Blumberg et al. 2013), and we have identified a series of new serine proteases and precursors that also play a role in this defense. R2RSP2 is one of many mosquito serine proteases and may play a role in amplifying the immune signal. Other serine proteases have also been shown to be vital for the melanization response in mosquitoes (Christophides et al. 2002), which can help to clear parasites; therefore, it is possible the R2RSRP2 increases melanization, although we have not measured this specific immune action. While ACEs have not been shown to have a direct effect on *Plasmodium* infection in mosquitoes, it has been suggested that they can cleave immune-related substrates and thereby alter immune activity. Our results demonstrate that ACEP has anti-*Plasmodium* activity in *An. stephensi* mosquitoes.

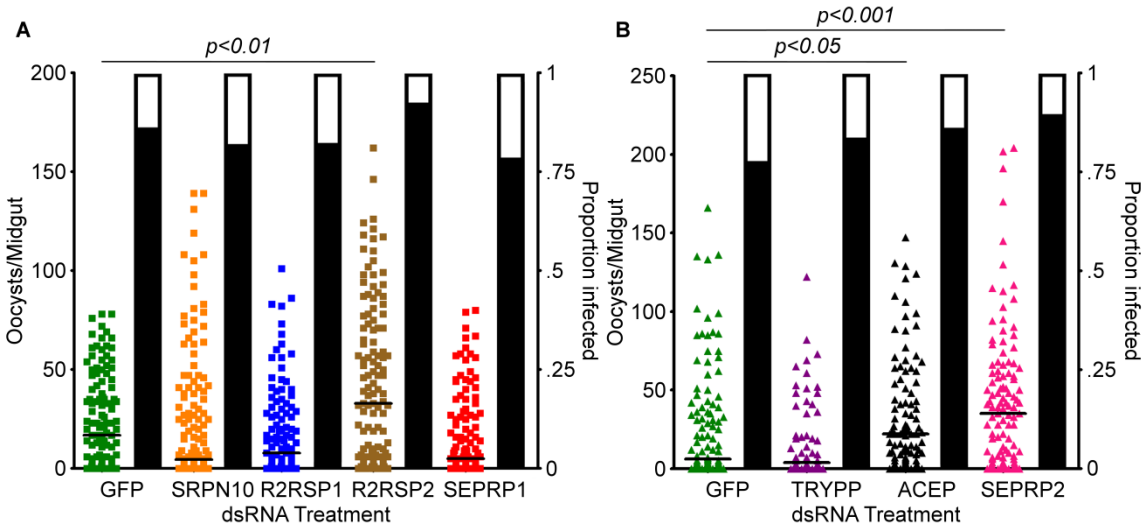

Figure 2.7: *P. falciparum* infection intensity following RNAi knockdown of protease genes. The number of oocysts per midgut of wild-type *A. stephensi* following RNAi-mediated depletion of: A) green fluorescent protein (GFP), serpin 10 (SRPN10), Rel2-responsive serine protease 1 (R2RSP1), Rel2-responsive serine protease 2 (R2RSP2), or serine protease precursor 1 (SEPRP1); and B) trypsin precursor (TRYPP), anigiotensin-converting enzyme precurser (ACEP), or serine protease precursor 2 (SEPRP2). Silencing of R2RSP2, ACEP, and SEPRP2 all led to significant increases in the number of oocysts per midgut. Each circle represents a single midgut, and horizontal black bars represent the median of the sample. Significance was determined by a Kruskal-Wallis test followed by Dunn's post-hoc test to compare immune-depleted mosquitoes to GFP controls. Significance was assessed at α=0.05. Supplementary data for this figure is given in table 2.2.

Further investigation is needed to determine whether this activity is a direct or indirect effect. Similarly, since SEPRP2 was identified as a precursor for an unknown serine protease, it is difficult to accurately predict all effects of altering the expression patterns of this gene, but, given the ability of serine proteases to act against *Plasmodium* discussed above, a precursor of any one of a number of serine proteases has the potential to be important for insect immunity.

*2.3.5 Effect of anti-*Plasmodium *effectors on midgut bacterial load*

In order to determine whether the genes identified previously as having an anti-*Plasmodium* effect act as general immune factors or are *Plasmodium*-specific, we tested them for involvement in controlling the mosquito midgut microbiota. The mosquito midgut is colonized by a variety of bacteria that need to be tightly controlled to prevent overproliferation and damage to the insect host (Pumpuni et al. 1996, Straif et al. 1998). Many different bacterial strains can be present in the mosquito gut, and the community varies from mosquito to mosquito and species to species; however, Gram-negative bacteria are considered to make up the majority of the species (Straif et al. 1998). Previous studies have implicated the IMD pathway as the main pathway involved in controlling the levels of bacteria in the mosquito midgut (Dong et al. 2009). Similarly, other studies have shown that the midgut microbiota is necessary to stimulate and prime the mosquito immune system and prepare it for future challenge (Dong et al. 2009, Clayton et al. 2012). Some anti-*Plasmodium* factors also act against the midgut microbiota, while others do not. Therefore, we tested our newly identified anti-*Plasmodium* effectors for an effect on the levels of bacteria in the mosquito midgut. The number of culturable bacteria per mosquito midgut was quantified by CFU assays following RNAi knockdown of potential novel anti-*Plasmodium* effector genes. Thus, we tested A2MRAP, LRTP, R2RSP2, ACEP, and SEPRP2 for anti-bacterial effect in both sugar-fed and blood-fed mosquitoes. In sugar-fed mosquitoes, one-way ANOVA showed a significant change in the log transformed number of colonies per midgut following knockdown of A2MRAP and LRTP (ANOVA $p<0.05$); however, a Dunnett's multiple comparison test showed no difference between either A2MRAP or LRTP and the GFP control, indicating that neither gene significantly affects midgut bacterial load

Figure 7.6						
	GFP	CD-36	BRP	NPC	A2M	LRTP
N	29	23	31	19	32	22
Range	0-114	0-88	0-148	0-108	0-191	0-127
Prevalence	75.86%	95.65%	97.10%	89.47%	93.75%	95.45%
Fisher's test *p* -value		0.0635	0.3271	0.2864	0.0723	0.1165
Median	9	11	21	15	56	71.5
% change		22.22%	133.33%	66.67%	522.22%	694.44%
Median no zeroes	18.5	12	31	16	63.5	73

Figure 7.7A					
	GFP	SRPN10	SERP42	SERP65	PREC65
N	127	142	111	126	120
Range	0-78	0-139	0-101	0-162	0-80
Prevalence	85.83%	81.69%	81.98%	92.06%	78.33%
Fisher's test *p* -value		0.4108	0.4795	0.1599	0.1367
Median	17	4.5	8	33	5
% change		-73.53%	-52.94%	94.12%	-70.59%
Median no zeroes	22	14	15	38.5	10.5

Figure 7.7B				
	GFP	TRYP	ANGI	PREC10
N	111	66	100	113
Range	0-166	0-122	0-147	0-204
Prevalence	77.47%	83.33%	86%	89.38%
Fisher's test *p* -value		0.4408	0.1548	0.0194
Median	6	4	22	35
% change		-33.33%	266.67%	483.33%

Table 2.2: Supplementary data for Figure 2.6 and 2.7. Includes the number of mosquitoes assayed, the range, prevalence, median and % change in the number of oocysts per mosquito midgut.

(Figure 2.8A). The ANOVA revealed no significant differences between GFP, R2RSP2, ACEP, and SEPRP2 in sugar-fed mosquitoes (Figure 2.8B). Following a blood meal, ANOVA revealed a highly significant difference in bacterial load between GFP,

A2MRAP, and LRTP (ANOVA $p<0.0001$), and a Dunnett's multiple comparison test showed a significant increase in bacterial load following knockdown of A2MRAP (Dunnett's $p<0.001$) (Figure 2.8C). As with the sugar meal, there was no significant difference in bacterial loads after knockdown of GFP, R2RSP2, ACEP, or SEPRP2 after a blood meal (Figure 2.8D).

The fact that none of the five genes tested had any effect on culturable bacteria levels in the sugar-fed mosquito midgut may indicate that the midgut microbiota is somewhat stable at this point. The midgut microbiota is adapted to the midgut environment and may therefore be able to evade action by various mosquito immune effectors. Alternatively, while multiple immune genes are able to affect the midgut microbiota, the genes we tested may display specificity for *Plasmodium* parasites and therefore not affect bacteria to a considerable degree. Specificity in mosquito immune response is common, since even pathogens in the same genus, such as *P. falciparum* and *P. berghei,* elicit strikingly different immune responses (Garver et al. 2009), and infection by the two is controlled by separate immune pathways. Thus, it is likely that highly divergent pathogens such as *Plasmodium* and bacteria would also be affected differently. Similarly, we saw no effect of AGBP1 on *Plasmodium* infection, although it has been shown to have an effect on *Staphylococcus aureus* infection in the mosquito (Dong et al. 2006b), adding credence to the theory of divergent immune action.

Following a blood meal, only A2MRAP had an effect on bacterial load. Blood meals have been shown in the past to have a large effect on the midgut microbiota of mosquitoes, leading to a large increase in the number of bacteria (Oliveira et al. 2011). This disturbance and proliferation may allow more opportunity for anti-bacterial genes to

take effect. Alternatively, the expansion in bacterial numbers following a blood meal may allow for dangerous levels of bacteria in the midgut that need to be controlled by the immune system in order to prevent damage to the mosquito. Alpha-2-macroglobulins have relatively broad binding specificities (Riordan 2003), which may explain why this protein can act against divergent pathogens such as *Plasmodium* and bacteria. Alternatively, since A2MRAP is an alpha-2-macroglobulin receptor-associated protein, it may be able to bind to multiple different alpha-2-macroglobulin receptors and therefore have a broad specificity based on the different combinations of alpha-2-macroglobulins and their receptors.

Our findings support the hypothesis that the mosquito's immune system is able to react in a specific manner to different pathogens, especially when they are distantly related. In addition, the fact that many more genes are up-regulated following Rel2 induction in the midgut than in the fat body implies that mosquito immune pathways exhibit tissue specificity and that the IMD pathway may be more important for immune defense in the midgut than in the fat body. The IMD pathway acts specifically against Gram-negative bacteria, which make up the majority of the mosquito midgut microbiota (Straif et al. 1998), and this pathway has been shown to be the major pathway involved in controlling the midgut microbiota in both mosquitoes and *Drosophila*. The greater expression of IMD pathway-responsive genes in the midgut than in the fat body may allow better control and a faster response to perturbations in the midgut microbiota.

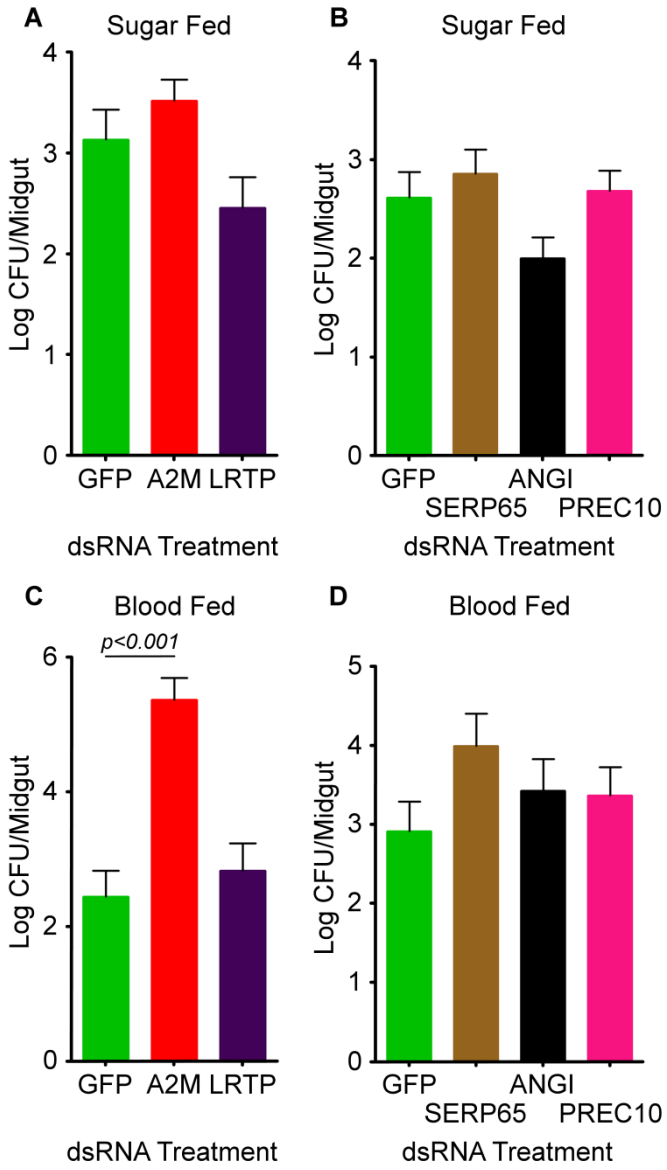

Figure 2.8: Influence of novel anti-*Plasmodium* genes on midgut microbiota. The number of colony forming units of culturable bacteria in the midguts of females following RNAi-mediated knockdown of genes shown above to have anti-*Plasmodium* effects. A) Sugar-fed mosquitoes following depletion of GFP, A2MRAP, and LRTP. B) Sugar-fed mosquitoes following depletion of GFP, R2RSP2, ACEP, and SEPRP2. C) Blood-fed mosquitoes following depletion of GFP, A2MRAP, and LRTP showed that A2MRAP depletion leads to a significant increase in CFUs per midgut. D) Blood-fed mosquitoes following depletion of GFP, R2RSP2, ACEP, and SEPRP2. Significance was determined by a one-way ANOVA, followed by Dunnett's multiple comparison test, with significance assessed at α=0.05. Bars represent the mean of three biological replicates, and error bars represent the standard error of the mean.

2.4 Conclusions

In this work, we have measured the changes in expression, at both the transcript and protein level, in the midguts and fat bodies of *P. falciparum*-resistant, genetically modified mosquitoes that are transiently overexpressing the IMD pathway-regulated transcription factor Rel2 (Dong et al. 2011). Through a combination of full-genome microarray-based expression analyses and iTRAQ proteomic analyses, we were able to see that specific up-regulation of Rel2 leads to differential regulation of a large number of both immune-related and other genes, including general immune genes and a large number of serine proteases cascade-related genes. We measured the effect of knockdown of multiple immune genes including serine protease cascade-related genes on *P. falciparum* infection in mosquitoes, thereby identifying a number of novel genes implicated in anti-*Plasmodium* defense. The presence of multiple serine proteases and their observed effect on *Plasmodium* infection indicate that these genes may be responsible for expanding and amplifying the IMD pathway signal and support the importance of serine proteases in the mosquito's immune defense. Of the five newly identified anti-*Plasmodium* genes, only one, A2MRAP, had an effect on the mosquito microbiota, and only after a blood meal. Finally, by observing significant changes at both the transcript and protein levels, we were able to look for any correlation between transcript and protein levels. In the midguts of our GM mosquitoes, we observed no correlation between the two, while in the fat body we saw a significant correlation, although many fewer genes were included in the fat body analysis. The lack of a strong correlation between transcripts and proteins concurs with the results of other studies that have observed a similar lack of correlation, and it likely indicates a large role for post-

transcriptional processing and control of translation, allowing mosquitoes to have a finer level of control over protein expression. Overall, our results indicate that Rel2 has a significant impact on both the mosquito transcriptome and proteome. The patterns of differential gene expression in the fat body were similar to those seen in transgenic *Ae. aegypti* overexpressing Rel2 under the VG promoter, indicating that these pathways may be well conserved across mosquito species (Zou et al. 2011). Also, as expected, we found that a number of genes controlled by the IMD pathway have an effect on anti-*Plasmodium* defenses, while others have no known immune function. This result indicates that the IMD pathway, and Rel2 specifically, controls or affects the expression of many non-immune processes. By means of these effects, the mosquito's immune response may be influenced by many factors that have not previously been considered to be part of canonical immune pathways. Similarly, induction of the mosquito immune system may alter many non-immune processes that can have far-reaching implications for mosquito fecundity and fitness. Overall, these results indicate that mosquito immune pathways act on a global level to produce complex changes in gene expression that will require further investigation to unravel fully.

Chapter 3: The effects of genetic modification on *An. stephensi* fitness

3.1 Introduction

Malaria is a global public health concern causing millions of cases each year and leading to hundreds of thousands of deaths, largely among children in sub-Saharan Africa (WHO 2014b). The *Plasmodium* spp. parasites that cause malaria are vectored by various mosquitoes in the genus *Anopheles*, and control of these vector mosquitoes has been employed as part of many malaria control programs. However, traditional vector control methods such as bed nets and insecticides have failed to bring about lasting changes in mosquito populations or reductions in malaria infection levels due to problems such as lack of compliance, difficulties in distribution and rising behavioral and physiological resistance in mosquito populations. Therefore, novel vector control methods are constantly being investigated, such the release of transgenic mosquitoes with reduced vector competence or breeding capacity and the use of the endosymbiotic bacterium *Wolbachia* to reduce the ability of the mosquitoes to spread disease or the number of mosquitoes in an area, respectively (Dong et al. 2011, Bian et al. 2013). However, despite the creation of many mosquito lines with reduced vector competence in various laboratories, there have been no widespread releases of such mosquitoes as part of a coordinated malaria control program, in part due to our lack of knowledge about how genetic engineering of mosquitoes affects their fitness.

Mosquitoes possess an innate immune system capable of responding to various invading pathogens including bacteria, viruses and eukaryotic parasites. This immune system comprises multiple pathways, such as the Toll and immune deficiency (IMD) pathways, which act to control different types of pathogens. The IMD pathway is

responsible for mosquitoes' resistance to the human malaria parasite *P. falciparum*, and our lab has created multiple mosquito lines that transiently over-express the IMD regulated NF-kB transcription factor Rel2 following a blood meal (Garver et al. 2009, Dong et al. 2011). One line, henceforth referred to as the CP15 line, up-regulates Rel2 in the midgut under the control of the carboxypeptidase promoter, while the other, designated the VG1 line, up-regulates Rel2 in the fat body under the vitellogenin promoter. These mosquitoes show greatly increased resistance to *P. falciparum* infection in the laboratory with limited effect on their fitness (Dong et al. 2011).

Our laboratory has also created multiple transgenic mosquito lines that transiently over-express specific splice forms of the immunity-related hyper-variable Down syndrome cell adhesion molecule (AgDSCAM) (Dong et al. 2012). The AgDSCAM gene can produce approximately 31,000 different splice forms that code for proteins with the ability to specifically bind to different substrates. Some of the splice forms target specific pathogens and mediate immune defense to those pathogens, giving the mosquito an immune specificity not unlike our own antibodies, despite the lack of an adaptive immune system (Dong et al. 2006a). Our laboratory has previously created mosquito lines transiently over-expressing both a long and a short form of *P. falciparum* specific AgDSCAM, containing the first 8 or 4 Ig domains, respectively, and has shown that they are highly resistant to *P. falciparum* infection (Dong et al. 2012). These lines use the same CP promoter as the previously mentioned CP15 line of Rel2 over-expressing mosquitoes and have a similar expression pattern. In this study we tested two lines bearing the DSCAM short form, referred to as DsPfs3 and DsPfs11, and one line bearing the long form, referred to as DsPfL8.

Despite the generation of *Plasmodium* resistant mosquito strains in both our laboratory and numerous others, no such mosquitoes have been released as part of a malaria control strategy. One possible obstacle for the successful deployment of this type of genetically modified mosquito is that the genetically modified organisms may be less fit than their wild-type conspecifics (Marrelli et al. 2006). Furthermore, there is an assumption that over-activating insect immune systems will lead to a decrease in fitness. There is precedence for both of these assumptions, as earlier experiments in *Drosophila* have supported this, however mounting evidence indicates this is not the case. Numerous early studies in *Drosophila* showed a negative fitness effect to immunity. Namely, infection with various bacteria and invasion by parasitoid wasps were shown to reduce fly lifespan, size and fecundity, especially in food limited conditions (Kraaijeveld et al. 2002, Zerofsky et al. 2005, McKean et al. 2008). However, these studies on the effects of immunity on fitness were often based on infection with bacteria, which can cause effects independent of the immune up-regulation. Early studies on the effects of transgenic insects focused on constitutively expressed genes, and showed some negative fitness effects (Catteruccia et al. 2003, Irvin et al. 2004). However, more recent studies on the effects of genetic modification and immunity on insect fitness have used inducible promoters to up-regulate immune gene expression independent of many confounding factors (Dong et al. 2011). These recent studies have exhibited less of a fitness effect than previous studies, but the field remains convoluted (Dong et al. 2011, Smith et al. 2013b).

In order to determine the general effects of genetic modification on mosquito fitness, we tested the fitness of 5 genetically modified *An. stephensi* lines under a variety of conditions. By testing these different lines we were able to compare effects due to

different inserts, different promoters and different insertion sites, making this a general study of the effects of genetic modification on mosquito fitness.

3.2 Materials and Methods

Mosquito Rearing

Wild-type *An. stephensi* (Liston) and transgenic mosquitoes of the CP15, VG1, DsPfs3, DsPfs11 and DsPfL8 lines were reared according to standard insectary conditions (Dong et al. 2011, Dong et al. 2012). Briefly, larvae were reared at low densities and fed a combination of ground fish flakes (Tetra) and cat food pellets (Purina). Upon emergence, adults were maintained on a 12h:12h light:dark cycle at 27º C with 80% humidity and provided constant access to a 10% sucrose solution in water, unless otherwise described for experimental conditions. To stimulate egg production, mosquitoes were provided a human blood meal from artificial membrane feeders on warmed water bottles. Genetically modified mosquitoes were screened for eye fluorescence each generation to ensure that all experimental mosquitoes bore the genetic modification.

Lifespan and Fecundity Measurements

In order to measure the lifespan of the various mosquito lines, adult mosquitoes were placed into cups within 12 hours of emergence. They were then held there until all mosquitoes died and the number of dead mosquitoes in the cup was recorded daily. For standard lifespan assays, mosquitoes were provided a sugar meal upon emergence and maintained until death. Mosquitoes were offered a blood meal 7 days after emergence for the single blood meal group, and at 7, 14 and 21 days after emergence for the multiple blood meal group. For both the single blood meal group and the multiple blood meal

group, only mosquitoes taking a blood meal at day 7 were kept for the rest of the study, while in the multiple blood meal group mosquitoes were kept even if they did not take a blood meal at days 14 and 21 post emergence. For starvation conditions, mosquitoes were allowed to emerge, provided a sugar meal for 3 days, then starved of sugar, but constantly provided a water soaked pad. For the blood fed group, on day 3 post emergence the mosquitoes were provided a human blood meal from artificial membrane feeders prior to starvation, while no such meal was provided for the sugar fed group. For mosquitoes kept at lower temperatures, the mosquitoes were reared as usual, but upon emergence the mosquitoes were moved to a 19º C chamber and maintained with constant access to a 10% sucrose solution until all mosquitoes had died. As with the standard conditions group, blood meals were provided for the applicable groups at days 7, 14 and 21 days post emergence. In all cases, once per week the dead mosquitoes were removed from the cups.

To measure fecundity, mosquitoes were reared under standard conditions and provided a blood meal 3 days post emergence. Mosquitoes were knocked down on ice immediately following the blood meal and any non-engorged mosquitoes were discarded. Two days after the blood meal, female mosquitoes were separated into individual vials containing moist filter paper and allowed to oviposit, and the number of eggs laid by each female was recorded. Only females that successfully took a blood meal during the first feeding and the second feeding were used for assays involving multiple blood meals. Following individual egg laying, eggs from the first blood meal were assayed for larval hatch rate, development time and male:female ratio. Eggs were hatched in trays as usual and the number of larvae recorded during the second instar. Larvae were maintained at

low density and the number of pupae present on each day was recorded to determine development time. Upon emergence, the number of male and female mosquitoes was counted.

Wing length

Adult wing length was used as a surrogate measurement for mosquito size. Wings were cut off 3 days post-emergence and placed on double sided tape on microscope slides. Pictures of wings were taken through a microscope objective containing a scale bar calibrated to a 1mm stage micrometer and measured using ImageJ.

Blood meal consumption

To measure the amount of protein consumed during a blood meal, a standard Bradfrod Assay was used. Mosquitoes were provided a human blood meal from artificial membrane feeders and allowed to feed for 30 minutes. At that time, the mosquitoes were immediately placed into a freezer and maintained at freezing temperatures until dissected. Mosquito midguts were dissected in sterile PBS and placed into tubes containing 50 ul of hypotonic buffer (5 mM Tris, 0.1 mM $MgCl_2$, 1 mM DTT, Protease inhibitors) with 1% triton. The midguts were then ground with a motorized pestle and incubated on ice for 30 minutes. The lysate was then put through two freeze/thaw cycles and spun at 15,000 g for 20 min at 4° C and 5 ul of the cleared lysate was added to 495 ul of 1x Quick-Start Bradford dye reagent (Bio-rad) and incubated at room temperature for 15 minutes. At this time, the samples were measured using a spectrophotometer and the amount of protein

recorded. Blood fed midguts were compared to the average of 10 unfed midguts, giving the amount of protein taken during a blood meal.

Recombinant insert location mapping

In order to determine the point at which the constructs inserted into the mosquito genome, a modified version of splinkerette PCR was performed, according to the modifications made in Smith et al. 2013 (Potter and Luo 2010). Briefly, genomic DNA from larval mosquitoes was cut with restriction enzymes and adapter sequences were ligated on. Two rounds of nested PCR using primers specific to the adapters or piggybac followed, and the PCR products were cloned into topoTA vectors and sequenced. The resultant sequences were located in the *An. stephensi* genome via a blast search and confirmed via PCR. PCR primers used are found in table 3.1.

Cage Competition Trials

To determine whether genetically modified mosquitoes are able to compete with their wild-type conspecifics, cage competition trials were set up. First, 50 wild-type and 50 genetically modified larvae were combined during the second instar and allowed to develop and emerge as usual. Upon emergence, the adult mosquitoes were maintained on either 10% sucrose (septic) or 10% sucrose with 100 units/mL of penicillin, 100 ug/mL of streptomycin and 75 ug/mL of gentamycin to remove their native midgut microflora (aseptic). Three days post-emergence, the adult mosquitoes were provided a human blood meal from artificial membrane feeders and allowed to oviposit. The resultant eggs were hatched and the proportion of transgenic larvae was recorded. To ensure that there were

Primer Name	Purpose	Sequence
Splink-bottom	splinkerette	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGG CTGAATGAGACTGGTGTCGACACTAGTGG
Splink-GATC	splinkerette	GATCCCACTAGTGTCGACACCAGTCTCTAATTT TTTTTTTCAAAAAAA
Splink-TAG	splinkerette	TAGCCACTAGTGTCGACACCAGTCTCTAATTTT TTTTTTCAAAAAAA
Splink-CGG	splinkerette	CGGCCACTAGTGTCGACACCAGTCTCTAATTTT TTTTTTCAAAAAAA
Splink-CCGG	splinkerette	CCGGCCACTAGTGTCGACACCAGTCTCTAATTT TTTTTTCAAAAAAA
Splink-AATT	splinkerette	AATTCCACTAGTGTCGACACCAGTCTCTAATTT TTTTTTCAAAAAAA
Splink-CATG	splinkerette	CATGCCACTAGTGTCGACACCAGTCTCTAATTT TTTTTTCAAAAAAA
Splink 1	splinkerette	CGAAGAGTAACCGTTGCTAGGAGAGACG
Splink 2	splinkerette	GTGGCTGAATGAGACTGGTGTCGAC
pBac LE 1	splinkerette	CAGTGACACTTACCGCATTGACAAGC
pBac LE 2	splinkerette	GCGACTGAGATGTCCTAAATGCAC
pBac RE 1	splinkerette	CGATATACAGACCGATAAAACACATGCGTC
pBac RE 2	splinkerette	ACGCATGATTATCTTTAACGTACGTCAC
CP15 F	GM testing	GTCGGCAAGGCTAAAGGAAC
CP15 R	GM testing	CGGTTCGGTCTTTAGTGTTAAGG
VG1 F	GM testing	CCACGGAAGCGTTAATGAGT
VG1 R	GM testing	GAGAGCGCGTTATTGTGTGA
DsPfs3 F	GM testing	CAAACGAGCAAGGAGACCTATATG
DsPfs2 R	GM testing	TGAGCTACTACGCTCCTAATCATG
DsPfs11 F	GM testing	GCTTCCGACGAAGTGGTAGA
DsPfs11 R	GM testing	AGCAGTTCGAATGGGTTCAC
DsPfL8 F	GM testing	CAATCGGATACCACAAATGTCCAG
DsPfL8 R	GM testing	CTCCATGACGCACATTCCTATTC

Table 3.1: PCR primers used in this study. "Gene name" displays the name of the gene targeted by the primer, "Primer type" indicates whether this primer was used for splinkerette PCR or detection of the insert in the mosquitoes and "Primer sequences" gives the sequences the primers.

no confounding effects of the mosquito larval population, the same experiment was set up except that the mosquitoes were reared at low densities and adults were allowed to emerge individually in the wells of a 24 well plate. These virgin adults were then combined in a ratio of 25:25:25:25 genetically modified males:genetically modified

females:wild-type males:wild-type females. Again, the mosquitoes were provided sucrose with or without antibiotics, provided a blood meal and allowed to oviposit, and the proportion of genetically modified mosquitoes was recorded at the larval stage of the next generation.

To determine whether the results of the initial breeding continued through multiple generations, the larvae from the two most successful lines (CP15 and DsPfs3) were allowed to develop to adulthood and maintained for 10 generations. Each generation, the proportion of genetically modified larvae was recorded and only 100 larvae were kept until the next generation in the same proportion as the total population. All larvae were allowed to develop to adulthood, and three days after the emergence of the last adult the mosquitoes were provided a human blood meal from artificial feeders, allowing the population to continue under Hardy-Weinberg equilibrium assumptions. At the 10^{th} generation after the initial cross, the mosquitoes were separated at the larval into wild-type or genetically modified larvae and allowed to develop into adults. These adults were provided a *P. falciparum* infectious blood meal, as described below, to determine their resistance to the parasite.

Insemination studies

To measure whether genetically modified males and wild-type males differ in their ability to inseminate females, 5 virgin genetically modified males and 5 virgin wild-type males were placed into a cup with 10 virgin wild-type females and provided sucrose with or without antibiotics, as described above. Three days later, the females were provided a blood meal and allowed to oviposit. The number of eggs per female was recorded, and

the eggs were hatched to determine whether the female was inseminated by a wild-type or genetically modified male. Any females that did not lay eggs were dissected in sterile PBS and their spermatheca removed for testing via PCR according to the procedures described in (Rogers et al. 2009). PCR primers are listed in table 3.1.

P. falciparum infections

To determine mosquito resistance to *P. falciparum* infection, mosquitoes were given infectious blood meals containing gametocytes from the NF54, HL1204 or 7g8 strain of *P. falciparum.* Mosquitoes were provided a blood meal of human blood containing the parasites from artificial membrane feeders and allowed to feed for 30 minutes before being maintained under standard conditions until dissection. 8 days after infection, mosquitoes were dissected in sterile PBS and their midguts stained with mercurochrome and the number of oocysts per midgut counted visually via light microscope.

Colony forming unit assays

To determine the number of culturable bacteria in the mosquito midguts, colony forming unit (CFU) assays were performed according to standard procedures. Briefly, adult mosquitoes were surface sterilized in 70% ethanol for 2-3 minutes, rinsed twice in sterile PBS and dissected in sterile PBS. Midguts were dissected out and placed into 150 ul of sterile PBS on ice, then ground with a mortar and pestle for 1 minute. The resultant solution was plated onto LB agar plates at 1:1 and 1:100 dilutions and allowed to grow at room temperature for 3 days, at which time the number of bacteria per plate was counted,

giving the number of bacteria per midgut. Dissections were performed prior to and 2 days post-blood meal, indicating the times when mating and oviposition occur, respectively.

O'nyong'nyong infections and plaque assays

GFP expressing O'nyong'nyong virus (ONNV) was obtained from the Foy lab. Frozen virus stocks were added to baby hamster kidney (BHK) cells and allowed to develop for 48 hours. At this point, 83 ul of the supernatant from the cell culture was added to 417 ul of human blood and provided as an infectious meal to 3 day old adult mosquitoes. 5 days after feeding, blood-fed mosquito midguts were dissected in sterile PBS and ground in 150 ul of DMEM containing 10% FBS and 110 units/mL of Penicillin and 110 ug/mL of streptomycin. These samples were used for plaque assays according to standard procedures, and the number of plaque forming units (PFUs) per midgut was recorded 7 days after plating.

Insecticide resistance testing

To measure the susceptibility or resistance of the mosquitoes to various insecticides, a standard World Health Organization (WHO) tube assay was performed, according to standard procedures. Supplies and insecticide-treated papers were obtained from the WHO. 3 day old adult mosquitoes were provided a human blood meal from artificial membrane feeders and tested for resistance to insecticides 1 hour post-blood meal. 25 blood fed adult females were exposed to each insecticide for 1 hour, and the number of knocked-down mosquitoes was recorded at 1 and 24 hours post exposure.

Wolbachia *infected mosquito crosses*

To test whether *Wolbachia* based malaria control strategies are compatible with our genetically modified mosquitoes, we crossed the genetically modified lines with the *Wolbachia* infected LB1 line from Zhiyong Xi (Bian et al. 2013). Because the lines are from the same background, we did not perform any backcrosses and tested the *P. falciparum* resistance in the offspring of the initial crosses. Three day old adult females were challenged with *P. falciparum* as described above.

3.3 Results and Discussion

3.3.1 Lifespan and fecundity

The lifespan of genetically modified *An. stephensi* strains were measured under a variety of different conditions for both female and male mosquitoes. Adult female mosquitoes subsist on nectar from various sources between blood meals, though the importance of these sugar meals is debatable. Therefore, we first tested the lifespan of female mosquitoes fed only on sugar (Figure 3.1A). When provided with a constant source of 10% sucrose but no blood meal, there was no difference in lifespan for any other mosquito strains tested. However, female mosquitoes that feed on only sugar cannot reproduce and are unable to spread malaria. Therefore, we performed similar experiments with mosquitoes provided with a blood meal (Figure 3.1B). Again, we observed no difference in the longevity of mosquitoes after being provided a blood meal seven days post emergence. Again, however, mosquitoes that take only one blood meal are unable to spread malaria, so we repeated the experiments a third time, this time providing the mosquitoes three separate blood meals, one every seven days (Figure 3.1C). When

provided three blood meals, female mosquitoes of the DsPfs11 line lived a significantly shorter period of time than wild-type females. Male mosquitoes only feed on sugar, as they do not need the protein provided by a blood meal to reproduce. Therefore, we tested the longevity of male mosquitoes only when provided with a sugar meal. As with females taking multiple blood meals, only DsPfs11 strain mosquitoes exhibited a significant decrease in longevity compared to wild-type mosquitoes (Figure 3.1D). Because DsPfs3 and DsPfs11 mosquitoes were generated using the same insert, but a fitness cost was only observed in the DsPfs11 strain, it is unlikely that the inserted gene is the cause of the effects.

Early experiments on genetically modified insects showed a marked reduction in lifespan (Catteruccia et al. 2003, Irvin et al. 2004). However, these experiments were based on insects with constitutively expressed transgenes, while our mosquitoes have transient induction of the transgene under blood-meal inducible promoters (Dong et al. 2011, Dong et al. 2012). Other groups have found similar results using inducible promoters, indicating that temporally restricted expression of genes may generally not cause a lifespan reduction in insects (Marrelli et al. 2007, Smith et al. 2013b). However, the fact that we observed a decrease in lifespan in one strain under certain feeding conditions indicates that it is important to test genetically modified mosquitoes, or other insects of interest, under a variety of conditions. If genetically modified organisms are only tested under one set of conditions, a potential fitness effect may not be observed. A decrease in lifespan could help or hinder the effectiveness of a genetically modified mosquito line. On the one hand, a lifespan reduction could make it harder for a mosquito line to invade the wild-type population. The longer a female mosquito lives, the more

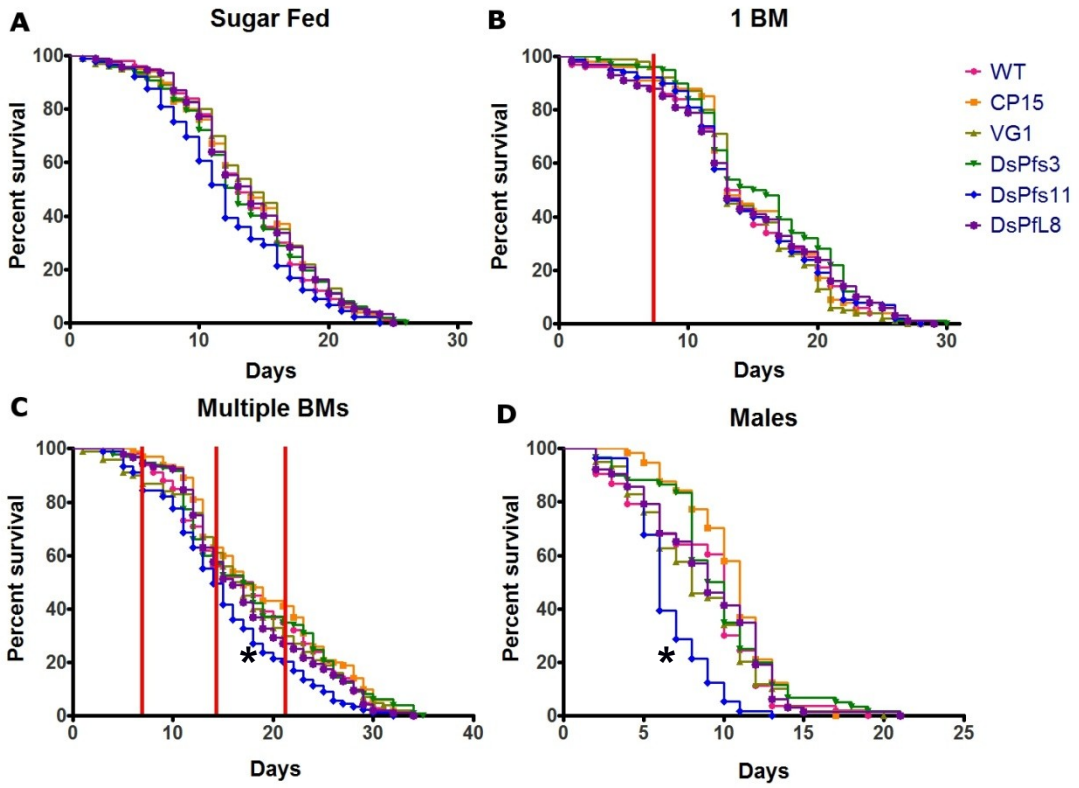

Figure 3.1: Lifespan of genetically modified mosquitoes. Female mosquitoes were fed on: A) sugar only B) sugar with a blood meal at 7 days post eclosion or C) sugar with 3 blood meals at 7, 14 and 21 days post eclosion, and the number of dead mosquitoes was recorded each day, as were D) male mosquitoes. There was no difference in the lifespan of genetically modified female mosquitoes fed on sugar only or provided only 1 blood meal when compared to wild-type mosquitoes of the same feeding status, however DsPfs11 females provided with 3 blood meals and DsPfs11 males lived a significantly shorter period of time than wild-type mosquitoes. All figures represent the pooled data from 3 separate replicates of 25 mosquitoes compared using a log-rank test with a = 0.05, and * indicates a significant difference in lifespan compared to wild-type mosquitoes. Vertical red lines indicate the days of blood feedings.

opportunities it has to lay eggs, which increases the population of mosquitoes. Therefore, if genetically modified mosquitoes die sooner, they may not be able to reproduce as many times, thereby laying fewer eggs and decreasing their ability to invade the population. Similarly, the fact that males dies sooner may give them fewer opportunities to mate, meaning that they do not inseminate as many wild-type females and decreasing the

ability of the genetically modified mosquitoes to replace their wild-type conspecifics. Conversely, a slight reduction in lifespan might not be sufficient to influence mating success in a significant fashion while further reducing the vectorial capacity of the mosquitoes. Because mosquitoes must live past the extrinsic incubation period before they can pass infectious parasites to the next host through their saliva, if they die sooner they may not have the opportunity to infect any humans. This effect would compound the decrease in vector competence caused by the genetic modification, making them more effective as a malaria control tool (Macdonald 1957). Nevertheless, whether the lifespan reduction decreases, increases or has no effect on the effectiveness of genetically modified mosquitoes as a tool for malaria control would have to be elucidated in the field.

Mosquitoes in the laboratory are provided with a constant source of sucrose, but this is not the case with mosquitoes in the wild. Sugar sources are widely dispersed and may be hard for mosquitoes to find, so we also tested the longevity of genetically modified mosquitoes when starved. Neither female mosquitoes provided with only sugar meals prior to starvation nor female mosquitoes provided a blood meal prior to starvation showed a significant difference in lifespan compared to wild-type mosquitoes for any strain (Figure 3.2). Again, this illustrates that it is important to test genetically modified organisms under a wide variety of conditions to ensure that any extant fitness costs are observed. In the field, mosquitoes may experience starvation conditions during the dry season or when plants are not abundant. As with a general lifespan reduction, the effect of this on the genetically modified mosquitoes' effectiveness as a malaria control tool would depend on the natural conditions. If starvation conditions dominate when the

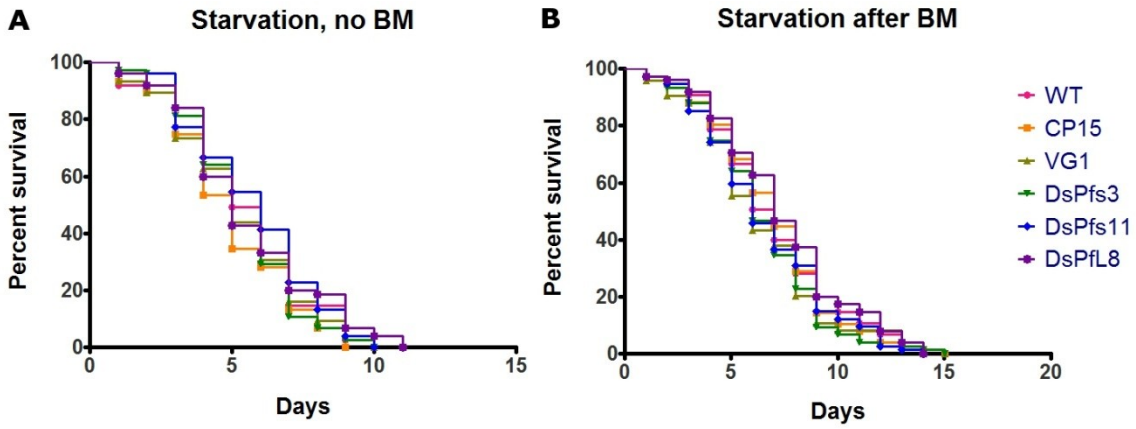

Figure 3.2: Lifespan of genetically modified mosquitoes when starved. Female mosquitoes were fed on: A) sugar only or B) a blood meal 3 days post eclosion before being starved and provided only water. The number of dead mosquitoes was recorded each day. There was no difference in the lifespan of female genetically modified mosquitoes compared to wild-type, regardless of whether they were provided a blood meal prior to starvation or not. Both figures represent the pooled data from 3 separate replicates of 25 mosquitoes compared using a log-rank test with a = 0.05.

mosquitoes were released, it could stop them from invading the population and make them less effective. However, if the mosquitoes were able to replace the wild-type population before starvation ensued, a decreased lifespan could cause a decrease in mosquitoes surviving past the extrinsic incubation period, as well as decreasing the total number of mosquitoes in the area, increasing the effectiveness of the control program. However, our mosquitoes showed no reduction in lifespan, indicating that there would be no effect of starvation on the effectiveness of our genetic modifications on malaria control.

Starvation is not the only adverse condition mosquitoes may face in the field. The mosquitoes in our insectary are maintained at a constant temperature of 27º C, while mosquitoes in the field may encounter a variety of temperatures. We, therefore, also tested mosquito lifespan at 19º C to see if their lifespan is affected by the lower temperature. Female mosquitoes, whether provided only sugar, one blood meal or

multiple blood meals, did not show a significant effect of temperature on lifespan, while male mosquitoes of the DsPfs11 strain did show a decreased lifespan at the lower temperature (figure 3.3). Temperature can have a profound effect on both the mosquito and the *Plasmodium* parasite, as temperature affects both the vector and the development time of the parasite in the mosquito (Blanford et al. 2013, Christiansen-Jucht et al. 2014, Murdock et al. 2014). The decreased survivorship observed in males could result in reduced mating capacity under adverse conditions, limiting the ability of this strain to invade wild-type populations. However, none of the other strains had any observable difference at this temperature, indicating that their ability to replace wild-type populations will not be adversely affected by lower temperatures. However, this study only considers populations held at a constant temperature, which is not the case in the field. Natural temperatures fluctuate through and between the days, and further experiments should be conducted under variable temperature conditions (Murdock et al. 2012).

The lifespan of mosquitoes is only one measure of their success. Additionally, the mosquitoes must be able to lay viable eggs and the resultant larvae must develop into adults for the mosquitoes to continue on to the next generation. Therefore, any genetically modified mosquitoes intended for field release must not be deficient in these areas in order to be successful. We measured the number of eggs laid by each strain of mosquito, along with the hatch rate, larval survival, development time and proportion of offspring that were female. When provided with only one blood meal, there was no difference in the number of eggs laid among any of the strains (Figure 3.4A). However,

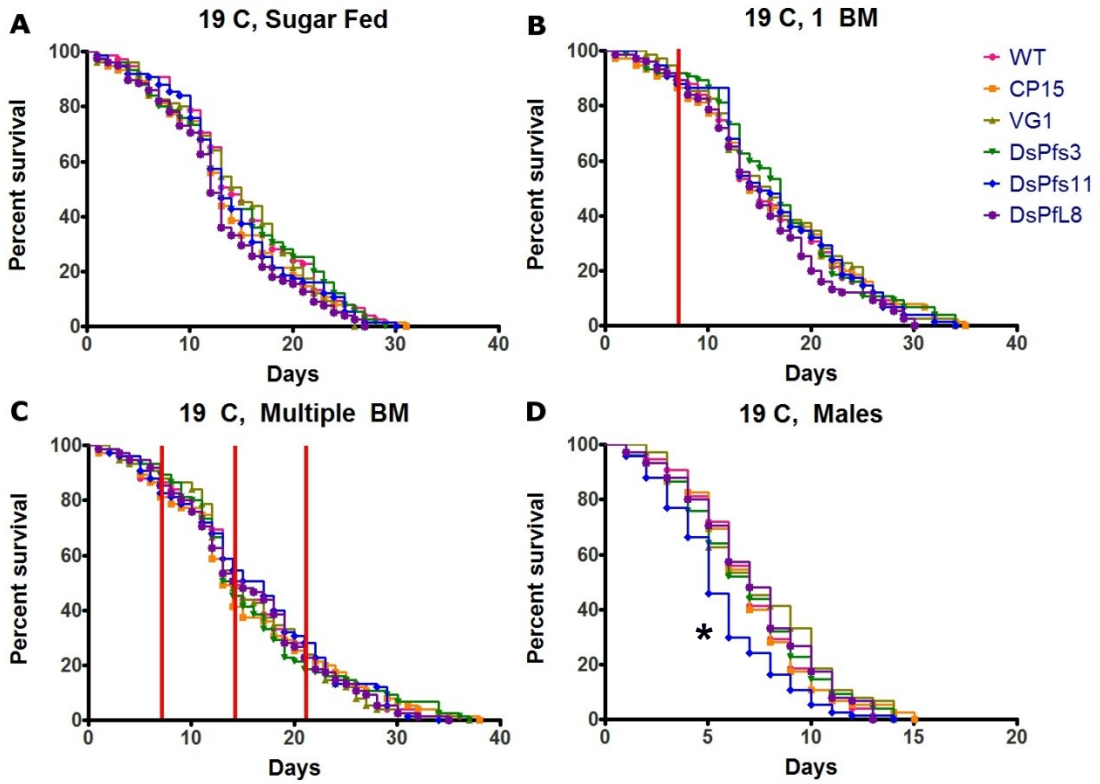

Figure 3.3: Lifespan of genetically modified mosquitoes at 19º C. Female mosquitoes were held at 19º C and fed on: A) sugar only B) sugar with a blood meal at 7 days post eclosion or C) sugar with 3 blood meals at 7, 14 and 21 days post eclosion, and the number of dead mosquitoes was recorded each day, as were D) male mosquitoes. There was no difference in the lifespan of female mosquitoes, regardless of feeding status or strain, however DsPfs11 males lived a significantly shorter period of time than wild-type males. All figures represent the pooled data from 3 separate replicates of 25 mosquitoes compared using a log-rank test with a = 0.05 and * indicates a significant difference in lifespan compared to wild-type mosquitoes. Vertical red lines indicate the days of blood feedings.

after taking a second blood meal DsPfs11 females laid significantly fewer eggs than wild-type females, while all other strains remained the same (Figure 3.4B). The same insert was used to create the DsPfs3 line, which had no such effect, indicating that these fitness costs, as with the lifespan effects exhibited by the DsPfs11 line, are not due to the construct itself. Following egg laying, the same proportion of eggs hatched for all tested strains (Figure 3.4C). There was no significant difference among the strains for

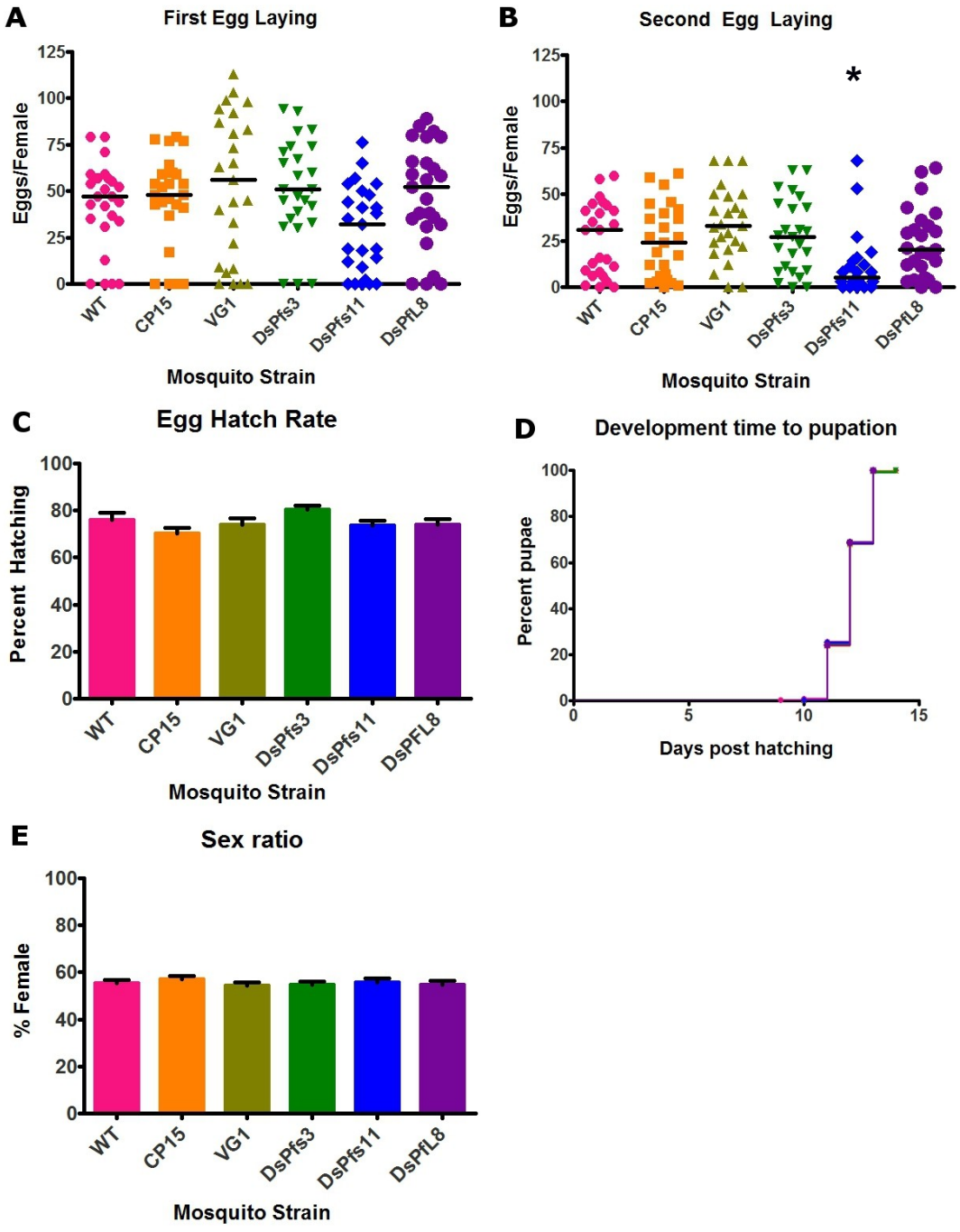

Figure 3.4: Fecundity of genetically modified mosquitoes. Female mosquitoes were provided A) 1 blood meal at 7 days post eclosion or B) two blood meals at 7 and 14 days post eclosion, and the number of eggs laid by individual females was counted after each feeding. The eggs from the first egg laying were hatched and C) the percentage of eggs hatching was recorded as was D) the development time of the resultant larvae and E) the sex ratio of the adults emerging from those larvae. There was no difference in the number of eggs laid after the first blood meal between any of the genetically modified mosquito lines and wild-type mosquitoes, however DsPfs11 females laid significantly fewer eggs

than their wild-type conspecifics following the second blood meal. There was also no difference in the egg hatch rate, development time or sex ratio between the strains tested here. All figures represent the pooled data from 3 separate replicates of the offspring from 10 mosquitoes compared using a Kruskal-Wallis test followed by a Dunn's post-hoc test (A, B, C, E) or a log-rank test (D) using a-0.05 and * indicates a significant difference in the number of eggs laid compared to wild-type mosquitoes.

development time either Figure (3.4D), nor was there a difference in the proportion of mosquitoes from each strain that were female or male (Figure 3.4E). Taken together, these data suggest that there is no significant difference in any of the main reproductive factors that we measured for any strain other than DsPfs11.

The reduction in the number of eggs laid by the DsPfs11 mosquitoes will severely limit their ability to replace wild-type mosquito populations. While some models have predicted complex interactions between larval population size and adult population size, it is generally believed that a reduction in the number of eggs laid will lead to fewer adults (Couret et al. 2014, Wasserberg et al. 2014). This means that there will be fewer genetically modified mosquitoes hatching, and therefore that the genetically modified mosquitoes will not replace the wild-type mosquito population. However, the other mosquito strains exhibited no such decrease in fecundity, indicating that they should be able to compete with wild-type mosquitoes, and that they may, therefore, be useful for malaria control programs.

3.3.2 Size and blood meal consumption

One factor that affects the ability of mosquitoes to mate and reproduce is their size (Sawadogo et al. 2013, Maiga et al. 2014). Larger females are able to lay more eggs and larger males may be more successful at mating. Male and female mosquitoes must match

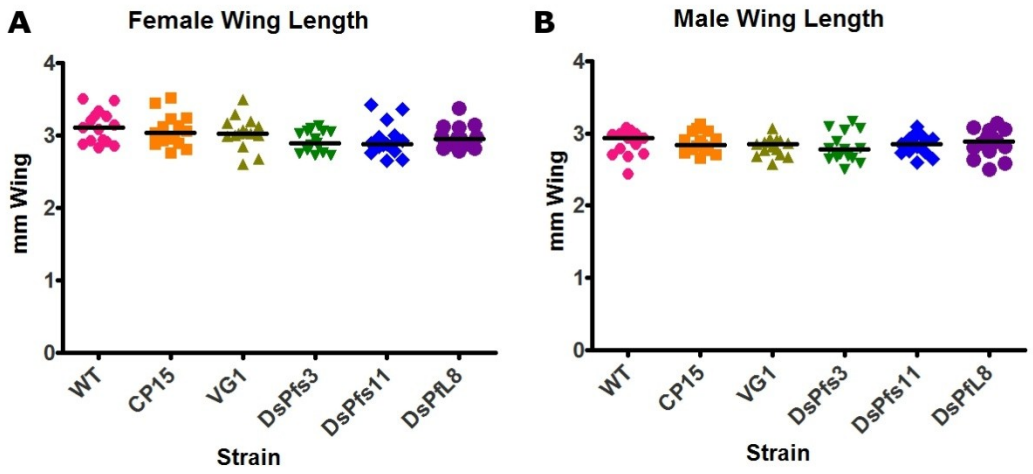

Figure 3.5: Size of genetically modified mosquitoes. The wing lengths of A) female and B) male adult mosquitoes were measured under a microscope using a micrometer. There is no difference in the length of wings from either female or male mosquitoes of any tested genetically modified strain compared to wild-type mosquitoes. Both figures represent the pooled data from 3 separate replicates of 5 mosquitoes compared using a Kruskal-Wallis test with a-0.05.

wing beat frequency to mate, and wing size affects the possible set of frequencies a mosquito can create, thereby affecting their mating success (Cator et al. 2009). To determine whether genetically modified mosquitoes were as large as wild-type mosquitoes, we measured the wing size of adult mosquitoes three days after emergence. There was no difference in wing size among and of the tested strains, which corroborates the previously observed data in which we saw no significant difference in fecundity between most of the strains. While the DsPfs11 strain did not have a difference in wing length in either males or females, they were not able to lay as many eggs. This, however, may be a post-mating effect, whereas wing length would cause a pre-mating effect. However, mate choice among mosquitoes is a complex and poorly understood system, and there may be other factors that affect it aside from wing length.

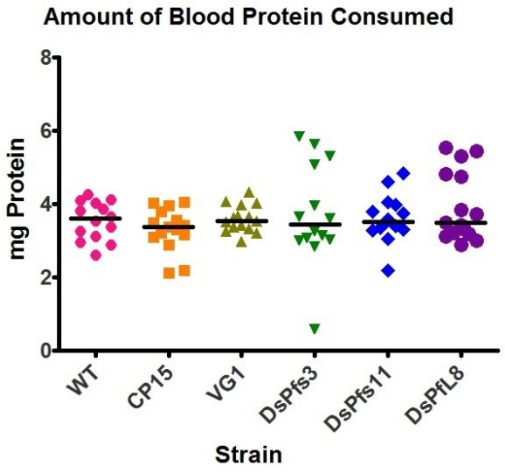

Figure 3.6: Amount of protein ingested during a blood meal by genetically modified mosquitoes. The amount of protein consumed by adult female mosquitoes in a blood meal take 3 days post eclosion was measured by a Bradford assay. There is no difference in the amount of blood protein consumed by genetically altered female mosquitoes compared to wild-type females. The figure represents the pooled data from 3 separate replicates of 5 mosquitoes compared using a Kruskal-Wallis test with a=0.05.

Female mosquitoes of the Rel2 over-expressing CP15 and VG1 lines have been shown previously to differentially regulate a large number of different genes compared to wild-type mosquitoes, including many not connected to the canonical immune pathways (Pike et al. 2014). This large and varied up-regulation could lead to a fitness cost due to the energy required to transcribe and translate the numerous genes. However, the data presented so far have not supported this. One potential reason for this would be additional energetic intake by taking a larger blood meal. We used a Bradford Assay to measure the amount of protein taken in during a blood meal in all of our mosquito strains. Standardizing blood fed midguts to the midguts of sugar fed mosquitoes and comparing between strains showed no difference in the amount of protein taken up by any of the genetically modified strains when compared to wild-type mosquitoes (Figure 3.6). The lack of a difference in the amount of protein taken up during a blood meal may partially

explain the lack of noticeable fitness consequences in most of our genetically modified lines. Because mosquitoes immediately after a blood meal have an abundance of proteins available and tend to rest while digesting the blood, any increased expression of genes may be offset by the abundance of energy available and lack of energy used for other activities, such as flying (Chang et al. 2008). Along with this, due to the blood meal inducible promoters used to drive transgene expression in our mosquitoes, the expression of the genetic modifications in our mosquitoes are very short term, so by the time the excess energy from the blood meal is expended, the expression has already returned to baseline. Therefore, while there may be greatly increased energy expenditure due to the genetic modification, the increased availability of proteins and energy may offset this expenditure.

3.3.3 Recombinant insert location mapping

One possible explanation for the large fitness cost observed in DsPfs11 mosquitoes and simultaneous lack of any observable fitness cost in DsPfs3 mosquitoes containing the same insert in a different location is position effects. These are effects on the expression or operation of genes due to an insertion happening in or near a gene or regulatory sequence. To determine whether position effects are the cause of the negative fitness effects in the DsPfs11 line we used splinkerette PCR to determine the insertion points of all the genetically modified mosquitoes strains (Potter and Luo 2010, Smith et al. 2013b). In three of the four lines that exhibit no fitness cost, the cassette did not insert near an annotated mosquito gene, while in DsPfs11 mosquitoes the genes inserted 2 kilobases bases downstream of a predicted cadherin gene. Cadherins are calcium

dependent adhesion molecules, and have been shown to be important in cell-cell interactions and adherence, as well as mosquito resistance to bacterial toxins, including the cry toxins of *Bacillus thuringiensis*. Therefore, disruption of the expression of this gene may cause dysfunction in cell-cell signaling, adherence or bacterial resistance, causing the observed fitness cost. Interestingly, the cassette of genes in the VG1 mosquitoes, which exhibit no fitness cost, inserted into a predicted odorant receptor. Odorant receptors form a large family of genes that bind odorants and are used in host-seeking and other olfaction-based orientation (Hallem et al. 2006). The insertion of the Rel2 cassette into this gene might, therefore, be expected to cause an inability of the mosquitoes to find hosts or oviposition sites, or interfere with similar olfaction-based activities. However, we did not observe any fitness cost of this insertion. This might be due to a number of factors. First, odorant receptors form a large family of genes, and there may be redundancy among them (Fox et al. 2001, Fox et al. 2002). Therefore, this gene may not be truly necessary, and interruption of it may not have an effect. Alternatively, because these mosquitoes are kept in small cages and provided both bloods and oviposition cups at very short range it is not necessary for long-range location of hosts or good breeding sites. We may have missed a fitness cost in these mosquitoes due to this short range, and such a cost would only be noticed in large cage or open-field trials where long-range seeking is necessary.

3.3.4 Cage population competition experiments

Because we saw no major fitness effects at either the lifespan or fecundity level of the genetically modified mosquitoes, we next decided to determine the competitiveness

of the genetically modified mosquitoes against their wild-type conspecifics in mixed cage populations. To do that, we crossed 50 genetically modified stage 2 larvae with 50 wild-type stage 2 larvae, allowed them to eclose, breed, take a blood meal and oviposit, then determined the proportion of the offspring that were genetically modified. These experiments were performed both with normal sugar fed mosquitoes and mosquitoes fed on sugar containing antibiotics to clear their midgut microbiota. Three of the five normal, septic, genetically modified strains showed the 75% genetically modified proportion of the population that would be expected under standard Hardy-Weinberg equilibrium (Figure 3.7A). However, both the DsPfs3 and CP15 lines exhibited increased numbers of genetically modified mosquitoes, with approximately 90% of the offspring being genetically modified. In mosquitoes treated with antibiotics, this effect disappeared and experiment using equal numbers of virgin male and female genetically modified or wild-type mosquitoes (Figure 3.7B). This yielded the same result, indicating that it was not an artifact due to different mosquito populations.

We next investigated whether this effect was limited to the first generation of mosquitoes or whether the proportion of genetically modified mosquitoes would either decrease or increase over multiple generations. To do this, we performed a cross using 50 genetically modified and 50 wild-type larvae again, but this time maintained the population for 10 generations. For each generation the larvae were screened to determine the proportion genetically modified, and only 100 larvae were kept, in order to maintain a constant population size, one of the necessary assumption for Hardy-Weinberg equilibrium. The larvae kept for each generation were chosen at the same proportion genetically modified as the total population, i.e. if 75% of the total population were

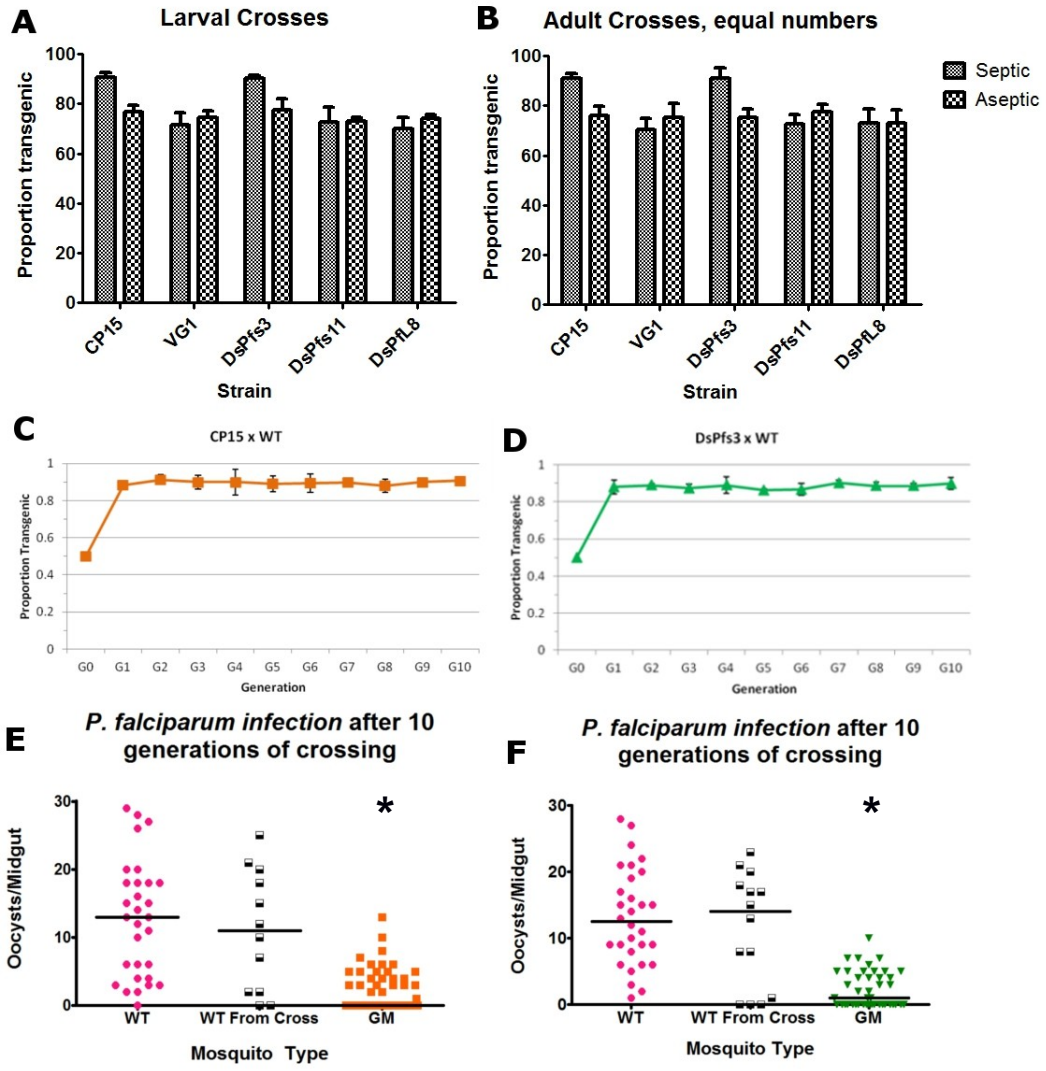

Figure 3.7: Crosses between genetically modified and wild-type mosquitoes. Crosses between wild-type and genetically modified mosquitoes were set up by combining A) 50:50 2nd instar wild-type:genetically modified larvae or B) 25:25:25:25 wild-type male:wild-type female:genetically modified male:genetically modified female virgin adults. The adult mosquitoes were maintained on 10% sucrose (small hatches) or 10% sucrose with antibiotics to remove the midgut microbiota (large checks), and the percent of genetically modified larvae from the f1 generation were recorded. Under both cross conditions, CP15 and DsPfs3 strain mosquitoes have higher than expected GM numbers in the f1 generation when containing the natural microbiota. Mosquitoes of the C) CP15 an D) DsPfs3 strains were maintained for 10 generations, showing that the increased proportion of genetically modified mosquitoes continues through multiple generations. At the 10th generation, the resistance of the resultant genetically modified mosquitoes were tested for resistance to *P. falciparum* and are still highly refractory to the parasite for both strains (E, F), when compared using a Kruskall-Wallis test followed by a Dunn's post-hoc test using a=0.05 and * indicates a significant difference in the number of oocysts per midgut compared to wild-type mosquitoes. Additional data in Table 3.2.

Figure 3.7E			
	WT	WT from Cross	CP15
N	30	12	52
Range	0-29	0-25	0-13
Prevalence	96.67%	83.33%	48.07%
Fisher's test *p* value		0.1916	0.0001
Median	13	11	0
% change		84.61%	0.00%
Median no zeroes	13	13.5	4
Figure 3.7F			
	WT	WT from Cross	DsPfs3
N	30	14	43
Range	1-28	0-23	0-10
Prevalence	100.00%	78.57%	53.48%
Fisher's test *p* value		0.0275	0.0001
Median	12.5	14	1
% change		112.00%	8.00%
Median no zeroes	12.5	17	4

Table 3.2: Supplementary data for Figure 3.7. Includes the number of mosquitoes assayed, the range, prevalence, median and % change in the number of oocysts per mosquito midgut.

genetically modified, 75% of the population maintained to the next generation were also genetically modified. After 10 generations, the proportion of mosquitoes bearing the genetic modification had not changed (Figure 3.7C, D). At the 10th generation we investigated whether the genetically modified mosquitoes were still resistant to *P. falciparum* infection, and observed that mosquitoes bearing the genetic modification were more resistant to *P. falciparum* infection than wild-type mosquitoes, whether from the

original wild-type population or the remaining 10% wild-type mosquitoes from the crosses (Figure 3.7E, F). This indicates that these mosquitoes may be ready for larger scale trials, as we observed that our mosquitoes were able to compete well with their wild-type conspecifics, and that doing so does not compromise their resistance to infection.

While this observation needs to be confirmed in large-scale cage or field trials, our results indicate that the genetically modified mosquitoes may be able to compete with and replace wild-type mosquito populations, and therefore be useful for malaria control. However, we cannot be certain that the two genetically modified strains that have a greater invasion than expected by Hardy-Weinberg equilibrium will do the same in the field. We observed that this effect is highly dependent on the mosquito microbiota, which will depend greatly on the environmental conditions under which the mosquitoes are reared. In the laboratory the microbiota is much more consistent than in the field, so these effects may not carry through to larger populations.

We also measured the proportion of wild-type female mosquitoes inseminated by wild-type or genetically modified male mosquitoes in competition trials. Female mosquitoes were allowed to mate with equal numbers of wild-type and genetically modified male mosquitoes, allowing them to choose their mates. These experiments were carried out with or without antibiotic treatment to remove the native microbiota. There was no significant difference in the percent of mosquitoes inseminated by genetically modified males between the strains of mosquitoes (figure 3.8). Nor was there a difference in the number of eggs laid by mosquitoes inseminated by genetically modified or wild-type males. The dynamics of mate choice in mosquitoes is poorly understood, and the

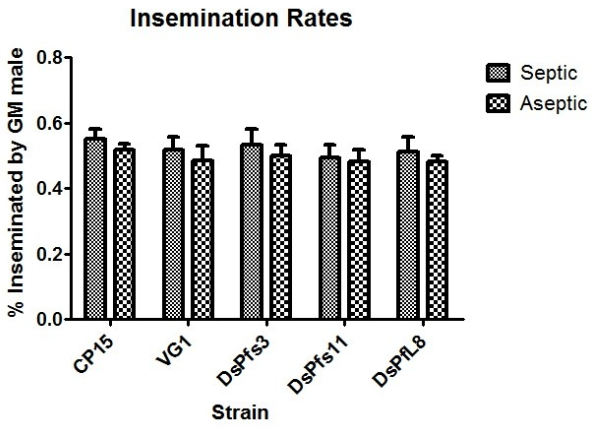

Figure 3.8: The insemination rate of wild-type females by wild-type or genetically modified males. Equal numbers of virgin wild-type and genetically modified males were allowed to mate with virgin wild-type females, and the proportion inseminated by genetically modified or wild-type males was recorded. There was no difference in the proportion of females inseminated between the groups, whether or not the mosquitoes were maintained on normal sucrose solution or sucrose solution containing antibiotics to remove the midgut microflora.

relationship between the midgut microbiota and mating has not been investigated in depth (Cator et al. 2009, Sanford et al. 2011). Further studies in this area, both with genetically modified mosquitoes and wild-type mosquitoes, are necessary to understand what might be causing these effects, and it is important to do so before releasing any genetically modified mosquitoes as part of a malaria control program, as mating dynamics will play a large role in the success of any population replacement program.

3.3.5 Control of the midgut microbiota

In order to determine whether genetically modified mosquitoes differ in their gut microbiota, we used colony forming unit (CFU) assays to measure the number of culturable bacteria found in mosquito midguts both before and after a blood meal, to observe the bacteria levels both when mosquitoes would be breeding and laying eggs. At

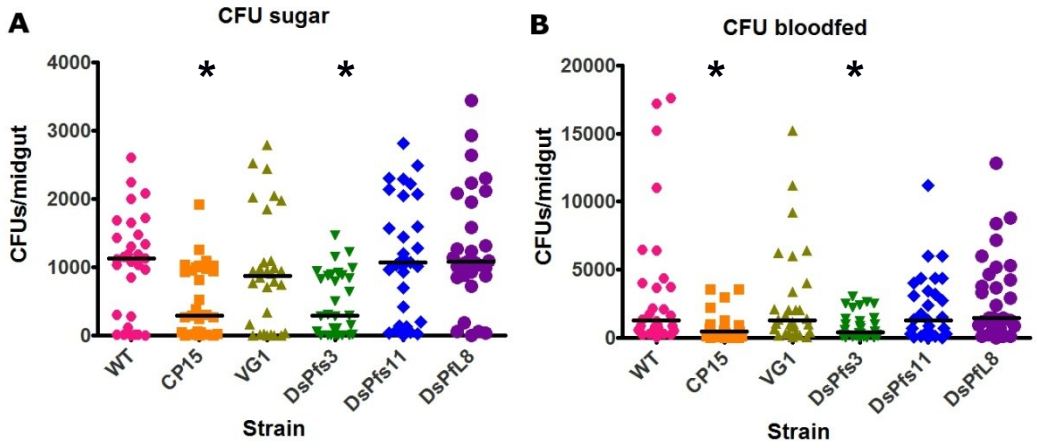

Figure 3.9: The number of culturable bacteria in genetically modified mosquito midguts. The number of culturable bacteria in adult female mosquito midguts was measured via colony forming unit assay in A) sugar fed females or B) blood fed females 2 days post blood meal. Under both feeding conditions, CP15 and DsPfs3 females have significantly fewer culturable bacteria in their midguts than wild-type females. Both graphs represent pooled data from 10 individual mosquitoes from 3 generations compared by a Kruskal-Wallis test followed by a Dunn's post-hoc test using a=0.05 and * indicates a significant difference in the number of culturable bacteria per midgut compared to wild-type mosquitoes.

both timepoints, we observed no significant difference in the number of culturable bacteria in the mosquito midgut in three of the strains, but the CP15 and DsPfs3 strains had significantly fewer culturable bacteria in their midguts than wild-type mosquitoes contained. This effect may be due to both where the transgene is expressed in the mosquito and how general the effect of the inserted gene is. Genes expressed in the midgut, including in all strains other than the VG1 line, are in position to affect microbes in the midgut. Genes expressed in the fat body may not be able to interact with the midgut microbiota, and therefore may not affect the number of bacteria found therein. This would explain why the CP15 line is able to reduce the number of culturable bacteria, while the VG1 line is not. Likewise, the effector molecule expressed by genetically

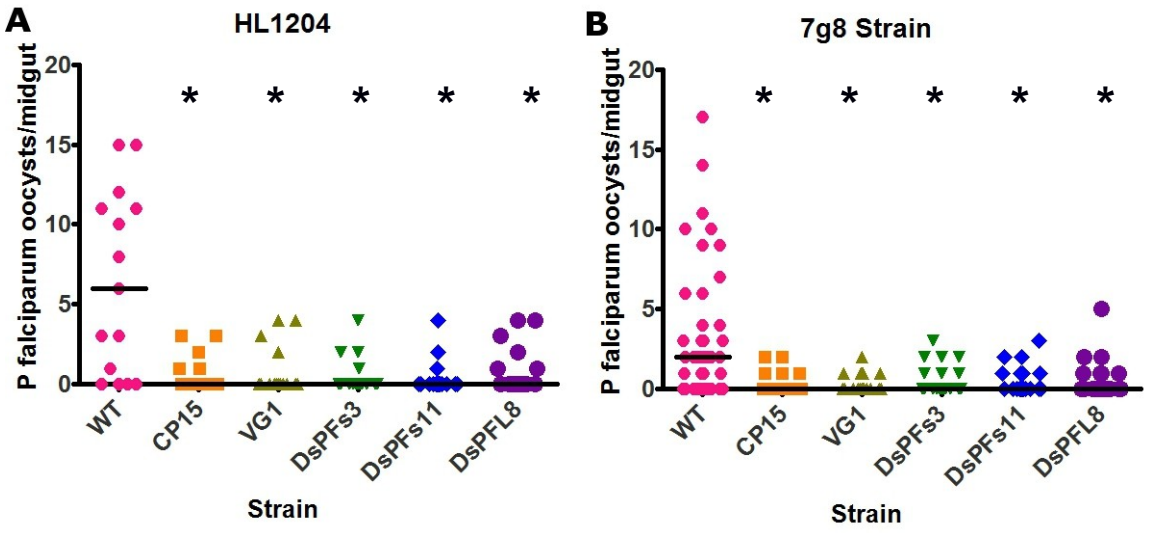

Figure 3.10: Resistance of genetically modified mosquitoes to multiple *P. falciparum* strains. Adult female mosquitoes were fed on infectious blood meals containing gametocytes from the A) Kenyan HL1204 and B) Brazilian 7g8 *P. falciparum* strains. All genetically modified mosquito strains tested exhibited increased resistance to both *P. falciparum* strains, indicating that these mosquitoes resist *P. falciparum* from multiple, geographically divergent, areas. Each figure represents mosquitoes compared by a Kruskal-Wallis test followed by a Dunn's post-hoc test using a=0.05, and * indicates a significant difference in the number of oocysts per midgut compared to wild-type mosquitoes. Additional data given in table 3.3

modified lines may be too specific to affect both bacteria and *Plasmodium.* Some of the many genes with expression altered by Rel2 in the CP15 lines also have activity against bacteria, allowing this line to control the number of bacteria in their midguts. Conversely, the DSCAM spliceform exhibited by the DsPfL8 line may bind too specifically to *P. falciparum* to be able to bind to off-target organisms such as the gut microbiota, while the short form of DSCAM found in the DsPfs3 line may have a broader specificity, leading to the reduction in the culturable bacteria in these mosquitoes' midguts.. This shows that the different genetically modified mosquito lines may have varied abilities to control their midgut microflora. As shown above, the mosquito microflora appears to influence mosquito reproductive patterns, while previous research has shown that the microflora

Figure 3.10A						
	WT	CP15	VG1	DsPfs3	DsPfs11	DsPfL8
N	15	15	15	15	15	15
Range	0-15	0-3	0-4	0-4	0-4	0-4
Prevalence	73.33%	33.33%	26.67%	26.67%	20.00%	40.00%
Fisher's test *p* -value		0.0656	0.0268	0.0268	0.0092	0.1394
Median	6	0	0	0	0	0
% change		0.00%	0.00%	0.00%	0.00%	0.00%
Median no zeroes	10	2	3.5	2	2	2.5
Figure 3.10B						
	WT	CP15	VG1	DsPfs3	DsPfs11	DsPfL8
N	43	29	19	38	17	27
Range	0-17	0-2	0-2	0-3	0-3	0-5
Prevalence	66.67%	27.59%	36.84%	39.47%	41.47%	33.33%
Fisher's test *p* -value		0.0001	0.0092	0.0018	0.0333	0.0011
Median	2	0	0	0	0	0
% change		0.00%	0.00%	0.00%	0.00%	0.00%
Median no zeroes	2.5	1	1	1	1	1

Table 3.3: Supplementary data for Figure 3.10. Includes the number of mosquitoes assayed, the range, prevalence, median and % change in the number of oocysts per mosquito midgut.

has a profound influence on mosquito resistance to *P. falciparum* and other pathogens (Dong et al. 2009). These reductions in the native microflora may be important for replacement of the wild-type population and control of the malaria parasite.

3.3.6 Resistance to various P. falciparum *strains*

Because previous experiments on our genetically modified mosquitoes only considered resistance to the NF54 line of *P. falciparum*, while there is considerable variation in *P. falciparum* strains worldwide, we decided to test the resistance of these

genetically modified mosquitoes against multiple *P. falciparum* lines from widely distributed geographic areas. We obtained two other strains of *P. falciparum* that would infect our mosquitoes, and tested the genetically modified strains for resistance to these. The genetically modified mosquitoes were highly resistant to both the Kenyan HL1204 and the Brazilian 7g8 strains, indicating that the mosquitoes can resist multiple strains of *P. falciparum* (Figure 3.9) (van Schalkwyk et al. 2013). While other laboratories have shown that the geographic distribution of both the mosquito and the parasite relative to each other, we have shown that our mosquitoes are able to resist infections from different areas (Molina-Cruz and Barillas-Mury 2014). While parasites from a given area may be able to evade the immune response in mosquitoes from other areas, they are not able to do so in our genetically modified mosquitoes. This is likely due to the large upregulation of the targeted genes, which may overwhelm the evasion of the parasites. Again, this indicates that these genetically modified mosquitoes may be a viable tool for malaria control, as the same effector mechanism could be used in multiple areas. Though different mosquito species would have to be modified for different geographic areas, the genes upregulated in our mosquitoes could be used in multiple areas, making the mosquitoes easier to create and the intervention easier to implement.

3.3.7 Resistance to O'nyong'nyong virus

Anopheles mosquitoes also act as the vector for the O'nyong'nyong virus, which causes a fever and symptoms similar to dengue fever. This virus has been spreading in recent years, and may continue to do so (Powers et al. 2000). Therefore, if genetically modified mosquitoes are to be implemented, it must be confirmed that they are not able

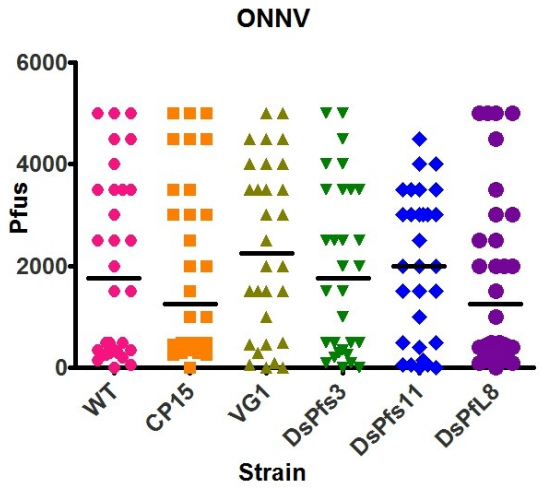

Figure 3.11: Resistance of genetically modified mosquitoes to ONNV. Adult female mosquitoes were provided an infectious blood meal containing O'nyong'nyong virus. Genetically modified mosquitoes showed no difference from wild-type mosquitoes in their ability to be infected with ONNV. This figure represents 10 mosquitoes from each of 3 generations compared by a Kruskal-Wallis test and a=0.05.

to spread this virus any better than wild-type mosquitoes. It is also possible that the genetically modified mosquitoes would resist this virus, making them an even more effective vector-borne disease control tool. To that end, we tested the ability of our mosquitoes to be infected by ONNV. All past studies on ONNV in mosquitoes have been performed in *An. gambiae,* but initial tests in our lab showed that *An. stephensi* mosquitoes can also be infected with the virus, albeit at a Lower rate (data not shown). Our genetically modified mosquitoes showed no difference in their ability to be infected with ONNV compared to wild-type mosquitoes (Figure 3.10). Past studies have not looked into the manner in which *An. gambiae* mosquitoes react to or resist ONNV infection, so we do not know the mechanism of resistance. However, other alphaviruses, such as dengue virus, are controlled largely by the Toll, JAK/STAT and RNAi pathways (Xi et al. 2008a, Souza-Neto et al. 2009, Jupatanakul et al. 2013). Our mosquitoes have

alterations to their IMD pathway or a specific immune gene, and therefore it is unlikely that they would be resistant to viruses.

3.3.8 Resistance to insecticides

If a wild-type mosquito population were replaced by a refractory population, the mosquitoes would still act as a nuisance to people. Therefore, people would still want to be able to remove or avoid being bitten by the mosquitoes as needed, so the mosquitoes would need to continue to be susceptible to insecticides. To ensure that this is the case, we tested the susceptibility of our mosquito strains to various insecticides from different classes. We chose to test the mosquitoes with the pyrethroid permethrin, the organophosphate malathion, the carbamate bendiocarb and the organochloride dichlorodiphenyltrichloroethane (DDT) using a standard WHO assay based on insecticide impregnated papers. Both 1 and 24 hours after exposure to the insecticides all mosquitoes were knocked down, while only a small proportion of the control mosquitoes were knocked down, indicating that the genetically modified mosquitoes are just as susceptible to insecticides as the wild-type mosquitoes. The fact that these mosquitoes are highly sensitive to insecticides, and that the genetic modifications do not increase their resistance to them would allow the mosquitoes to be removed from the field if necessary, as well as allowing these mosquitoes to be used in areas currently employing long lasting insecticide treated bednets or indoor residual spraying. These currently implemented vector control methods would not need to be stopped, nor would they lose effectiveness after these genetically modified mosquitoes were released, which would likely aid in public acceptance of the novel interventions.

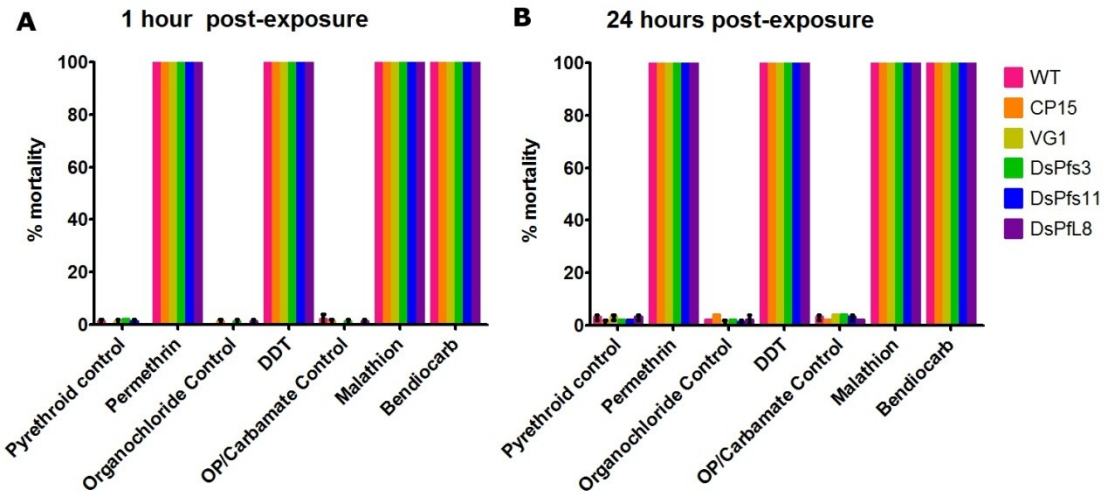

Figure 3.12: Resistance of genetically modified mosquitoes to insecticides. Blood fed adult female mosquitoes were exposed to various insecticides for 1 hour immediately following a blood meal, and their survival was recorded A) 1 or B) 24 hours post exposure. Genetically modified mosquitoes showed no difference from wild-type mosquitoes in their resistance to various insecticides. Figures represents 25 mosquitoes from each of 3 generations.

3.3.9 Compatibility with Wolbachia *infection*

Another intervention that has been suggested to control vector-borne diseases is the use of the intracellular bacteria *Wolbachia.* This bacteria is a reproductive parasite which uses modification of its host reproductive system to quickly spread to fixation in a population (Sinkins 2004). While not naturally found in many common disease vectors, such as *Aedes aegypti* or *Anopheles* spp. mosquitoes in general, it is found in approximately 75% of insect species and numerous other arthropods, showing different reproductive phenotypes depending on its host (Jeyaprakash and Hoy 2000). Due to its ability to spread in a population, this bacteria was initially suggested as a gene driver to spread a genetically modified mosquito population into a wild-type population (Sinkins and Godfray 2004). However, later experiments showed that infection with *Wolbachia* can act as an anti-parasite effector by itself, as it reduces infection with various pathogens, including *P. falciparum* and multiple viruses (Hedges et al. 2008, Bian et al.

2010, Bian et al. 2013). Therefore, *Wolbachia* is now being considered as a standalone disease intervention, and *Wolbachia* infected *Ae. aegypti* are being deployed to combat dengue virus in numerous locations around the world (Hoffmann et al. 2011). While *Anopheles* spp. mosquitoes have only recently been infected with *Wolbachia* and are still far from being used to fight *Plasmodium* infections, they do show significantly reduced infection with *P. falciparum* (Hughes et al. 2011, Bian et al. 2013). These *Wolbachia* infected *Anopheles* mosquitoes may one day be used to control malaria, as may genetically modified mosquitoes. If this is the case, the two cannot be incompatible, as their distributions may overlap. Likewise, the two interventions could be combined to increase their resistance to infection or utilize *Wolbachia* as a gene driver for the genetically modified mosquitoes. However, *Wolbachia* are gram negative bacteria, which are generally controlled by the IMD pathway. Therefore, our genetically modified mosquitoes may affect *Wolbachia* infection levels. *Wolbachia* densities have been shown to correlate with the effectiveness of the reproductive modifications caused by *Wolbachia*, so a decrease in bacterial densities could limit the effectiveness of the intervention (Mouton et al. 2006, Duron et al. 2007).

We tested the *Plasmodium* resistance of crosses between genetically modified and *Wolbachia* infected mosquitoes. By crossing virgin *Wolbachia* infected female mosquitoes with virgin genetically modified male mosquitoes, we created genetically modified mosquitoes that were simultaneously infected with *Wolbachia*. Females of the f1 generation were tested for their resistance to *P. falciparum* infection, and we saw no difference in the ability of *Wolbachia* infected genetically modified mosquitoes to resist *P. falciparum* relative to either single interventions at either a low or high infection level.

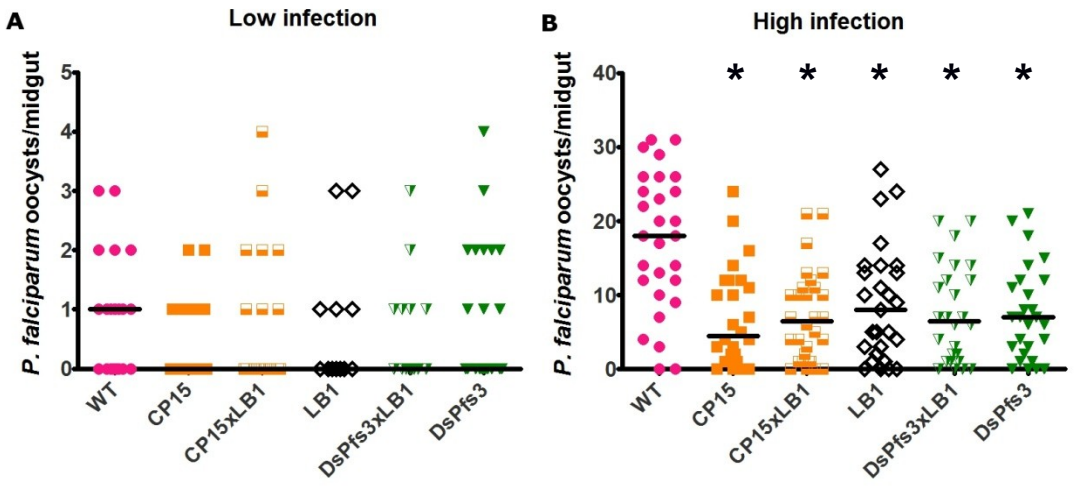

Figure 3.13: Resistance of genetically modified mosquitoes to *P. falciparum* after crossing with *Wolbachia* infected lines. Genetically modified mosquitoes were crossed with *Wolbachia* infected mosquitoes to create lines that were both genetically modified and *Wolbachia* infected and provided a *P. falciparum* infectious blood meal at a A) low infection level or B) high infection level. All lines had fewer oocysts on their midguts than wild-type mosquitoes when infected at a high level, but mosquitoes bearing both the genetic modification and the *Wolbachia* infection did not differ in their *P. falciparum* resistance from genetically modified or *Wolbachia* infected mosquitoes alone, regardless of infection level. Strains were compared using a Kruskal-Wallis test and Dunn's post-hoc test with a=0.05, and * indicates a significant difference in the number of oocysts per midgut compared to wild-type mosquitoes. Additional data in table 3.4.

We initially tested infection at a low level, to mimic the low levels of oocysts found in mosquitoes in nature. However, this low level of infection may be so low as to mask any additional decrease in infection when the two lines are crossed. Therefore, we repeated the experiment with a higher level of infectious parasites in the blood meal, but saw the same result. This indicates that there is no additive effect to combining the two interventions, but likewise that there is no negative effect to combining them. Therefore, the two interventions could be deployed in the same geographic area without hindering resistance to *P. falciparum*. As with insecticide susceptibility, the compatibility of genetically modified mosquitoes with *Wolbachia* infected mosquitoes could be important for the control of *P. falciparum*.

Figure 3.13A						
	WT	CP15	CP15 x LB1	LB1	DsPfs3 x LB1	DsPfs3
N	21	22	19	12	14	21
Range	0-3	0-2	0-4	0-3	0-3	0-4
Prevalence	57.14%	40.90%	42.10%	41.67%	42.86%	47.62%
Fisher's test *p*-value		0.366	0.5273	0.4813	0.4998	0.7579
Median	1	0	0	0	0	0
% change		0.00%	0.00%	0.00%	0.00%	0.00%
Median no zeroes	1	1	2	1	1	2
Figure 3.13B						
	WT	CP15	CP15 x LB1	LB1	DsPfs3 x LB1	DsPfs3
N	28	26	30	27	26	27
Range	0-31	0-24	0-21	0-27	0-20	0-21
Prevalence	9284.00%	84.62%	86.67%	85.19%	84.62%	85.19%
Fisher's test *p*-value		0.4126	0.6714	0.4216	0.4126	0.4216
Median	18	4.5	6.5	8	6.5	7
% change		25.00%	36.11%	44.44%	36.11%	38.88%
Median no zeroes	19	6.5	7	10	7	7

Table 3.3: Supplementary data for Figure 3.10. Includes the number of mosquitoes assayed, the range, prevalence, median and % change in the number of oocysts per mosquito midgut.

3.4 Conclusions

While many people have assumed that there will be a fitness cost associated with any genetic modification, and that this will interfere with the ability of genetically modified mosquitoes to be used for malaria control, we did not find evidence of this under laboratory insectary conditions. We tested five separate genetically modified mosquito lines and only found evidence for decreased fitness in one line. When considering mosquito lifespan, size, fecundity and many other variables there was only a

fitness cost in one strain, which can be explained as a position effect. In the one case with a fitness effect, the construct inserted just 3' of a gene, and the same construct inserted into a different location experienced no noticeable effect. Together, this indicates that there is no inherent cost to genetic modification in mosquitoes, though some strains may experience one. While these studies are limited to laboratory experiments, there is nothing to indicate that the mosquitoes should not be used in larger scale cage or field experiments, and there is no reason to believe that these mosquitoes would not be able to form an important part of malaria control programs in the future. However, these tests were all performed under standard laboratory conditions, and not all findings presented herein may apply under field conditions. Conditions such as fluctuating temperatures, host seeking and the large spatial scale present in the field may exacerbate or expose unobserved fitness effects. Also, while our mosquitoes appear fit, either a genetic drive mechanism or a reduction in the wild-type population followed by inundative releases would be necessary to drive the genetically modified mosquitoes to fixation. Similarly, for any genetically modified organism to be distributed on a large scale requires both governmental and popular support, and may encounter resistance. Finally, these mosquitoes are not fully resistant to *P. falciparum* infection, and greater resistance should be sought before these mosquitoes are employed as part of a large scale malaria control program. Despite these limitations thus far, genetically modified mosquitoes show no inherent fitness costs and further work should be done to improve their effectiveness. Similarly, large-scale field trials should be performed to determine how these mosquitoes perform on a large spatial scale. This will allow us to evaluate the effectiveness of these mosquitoes to control malaria, and allow us to make informed decisions about their use.

The data presented in this study indicate that genetically modified mosquitoes are a viable tool for malaria control programs.

Chapter 4: Conclusions and future directions

The recent availability of the *An. gambiae* and *An. stephensi* genomes have allowed great leaps in the knowledge of these two vector species and their interactions with the human malaria parasite. This genomic information has allowed researchers to determine the important mosquito immune pathways responsible for resistance to the *Plasmodium* spp. parasites responsible for malaria in humans. Further, this knowledge, combined with recent advances in the genetic engineering of mosquitoes, has led to the ability of multiple laboratories to create genetically modified mosquitoes with increased resistance to *Plasmodium* infection. Our lab is one that has created *P. falciparum* refractory mosquitoes, though these mosquitoes have not yet been deployed in the field.

We used two *P. falciparum* resistant *An. stephensi* lines that over-express the Rel2 associated transcription factor Rel2 upon taking a blood meal to elucidate the genes under the control of the IMD immune pathway in *An. stephensi* mosquitoes. The IMD pathway is one of the main immune pathways responsible for mosquito resistance to gram-negative bacteria and *P. falciparum*, and is a good target for creating mosquitoes resistant to the human malaria parasite. By studying the global transcriptomic and proteomic effects of Rel2 overexpression we have identified a large number of genes under the control of Rel2. Examining these genes further has shown that a large number of genes both within and outside the canonical immune pathways are controlled by Rel2, indicating that this transcription factor has wide-ranging effects on mosquito gene control. Further, we were able to identify numerous novel mosquito genes responsible for resistance to the human malaria parasite.

While many genetically modified mosquito strains resistant to *P. falciparum* infection have been created by a variety of labs, none have been employed as a part of a widespread malaria control program. While a variety of factors influence this lack of implementation, one major contributing factor is that people often assume that genetically modified organisms will carry an inherent fitness cost. We tested 5 separate genetically modified mosquito lines under a variety of conditions and observed no fitness cost in 4 of the lines. The 5th line, which did bear a fitness cost, showed that the gene cassette inserted near another gene, indicating that the fitness effects were due to a position effect, and not the act of genetic modification itself, as an identical copy of the same insert at a different location in another line did not impose a fitness cost. Furthermore, all tested mosquitoes were resistant to various *P. falciparum* strains, but showed no increased resistance to other pathogens or insecticides, nor any negative interactions with the bacterium *Wolbachia*. This indicates that genetically modified mosquitoes can form a part of future malaria control programs without large negative effects. While all experiments were performed in laboratory settings, there is no evidence that further tests on these lines, including larger cage and field trials should not be pursued. Because there was no fitness effect in the laboratory shows that these genetically modified mosquito strains are ready for much larger-scale trials and, potentially, implementation as part of a malaria control program.

However, much work remains to be done before these mosquitoes can be deployed in the field. Notably, the fact that all these tests were done in the laboratory means that there may be unforeseen effects of genetic modification in the field. While most of our strains had no negative fitness effects in the laboratory, the large number of

differentially-regulated genes in the Rel2 mosquitoes indicates that there is a large energy expenditure associated with the genetic modifications, and that a fitness effect is possible. These effects may not be noticed until deployment in the field, and therefore field trials are important prior to large scale releases.

Even if these mosquitoes are completely successful in the field, there are still many barriers between genetically modified mosquito releases and full malaria eradication. For example, these mosquitoes are not fully resistant to *Plasmodium* infection. Models indicate that in areas with high levels of malaria transmission full resistance and full replacement are necessary for control. Therefore, mosquitoes with greater resistance must be developed. Similarly, mosquitoes with an adequate gene driver or strategies for gene driver-independent releases are necessary to allow the refractory genetically modified mosquitoes to replace the wild-type mosquitoes. Before any release of genetically modified organisms can proceed, public support of the release must be gathered. While there have been some releases of genetically modified insects and other organisms, widespread acceptance of genetic modification has not yet occurred, and this remains an area ready for research and policy-based advances. There are also other pathogens spread by *Anopheles* mosquitoes, such as *Wuchereria bancrofti* against which these mosquitoes should be tested. Finally, any release will require a facility to rear a large number of mosquitoes to be released. Recent advances in mosquito rearing and screening have made progress toward this goal, but are not yet ready for full implementation. Generally, therefore, while genetically modified mosquitoes are ready for the next step toward implementation, they must be tested on a small scale before being released and any intervention must be slowly scaled up.

We have shown that, despite the great increase in global gene transcription and translation in genetically modified mosquitoes, there is no reason to believe that genetic modification of mosquitoes based on transient expression of the gene of interest following a blood meal will lead to a fitness detriment. Our mosquitoes showed negative fitness costs only when the gene integrated near another gene, a condition that is easy to detect. This means that any genetically modified mosquitoes meant for vector-borne disease control should be screened for these effects, but that they are not inherent to the system. Therefore, genetically modified mosquitoes remain a viable tool for malaria control and further studies should be pursued to test these mosquitoes on a larger scale.

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EDUCATION

Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD, 2010-Present
Ph.D. in Molecular Microbiology and Immunology

Michigan State University, East Lansing, MI, 2008-2010
M.S. in Entomology. Thesis Title: "Three way interactions between *Wolbachia*, dengue virus, and their host, *Aedes aegypti*"

Oberlin College, Oberlin, OH, 2004-2008
B.A. Majors: Biology and Mathematics, Honors in Mathematics
Minors: Computer Science and Religion

AWARDS

The Dr. Lloyd and Mae Rozeboom Scholarship: Award for the study of Medical Entomology, Spring 2011 and Spring 2012

Hope Hibbard Memorial Scholarship in Biology: Scholarship for continued research in biology following graduation, Spring 2008

Edward Wong Memorial Prize in Mathematics: Award for quality research in mathematics, Spring 2008

Norman J. Goldring Scholar-Athlete Award: For the four-year athlete with the highest GPA during his/her Oberlin career, Spring 2008

Elected as Associate member, Sigma Xi, Spring 2008

PUBLICATIONS

Sandiford, S.L., Y. Dong, **A. Pike**, B.J. Blumberg, A.C. Bahia, and G. Dimopoulos. 20XX. "Actin is an extracellular insect immune factor." In review.

Pike, A., A. Vadlamani, S.L. Sandiford, A. Gacita and G. Dimopoulos. 2014. "The Rel2-regulated transcriptome and proteome of *Anopheles stephensi." Insect Biochemistry and Molecular Biology* 52:82-93.

Hamm, C.A., C.A. Handley, **A. Pike**, M.L. Forister, J.A. Fordyce, and C.C. Nice. 2014. "*Wolbachia* infection and Lepidoptera of conservation concern." *Journal of Insect Science* 14.6.

Pike, A., C.M. Cirimotich and G. Dimopoulos. 2013. "Impact of Transgenic Immune Deployment on Mosquito Fitness." *In* W. Takken and C.J. Koenraadt [eds.], Ecology of Parasite-Vector Interactions. Wageningen Academic Publishers, Wageningen. 2013.

Dong, Y., C. M. Cirimotich, **A. Pike**, R. Chandra and G. Dimopoulos. 2012. *Anopheles* NF-κB-regulated splicing factors direct AgDscam-mediated pathogen-specific immunity. *Cell Host and Microbe* 12(4):521-30.

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Guo, X., Y. Xu, G. Bian, **A. Pike**, Y Xie, and Z. Xi. 2010. Response of the mosquito protein interaction network to dengue infection. *BMC Genomics* 11:380.

Bosch, R. and **A. Pike**. 2009. "Map-Colored Mosaics" *Bridges Banff II: Mathematical Connections in art, music, and science*. 139-146.

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POSTER PRESENTATIONS

Pike, A. and G. Dimopoulos. "Transgenic *Anopheles stephensi* fitness and susceptibility to various infections," poster presented as part of the American Society for Tropical Medicine and Hygiene Annual Meeting, New Orleans, LA, USA 2-6 November 2014.

Pike, A. and G. Dimopoulos. "The effects of transient immune deployment on transgenic *Anopheles stephensi* fitness," poster presented as part of the American Society for Tropical Medicine and Hygiene Annual Meeting, Washington, DC, USA 13-17 November 2013.

Pike, A. and G. Dimopoulos. "The effects of transient immune deployment on transgenic *Anopheles stephensi* fitness," poster presented as part of the ESF/EMBO symposium on Integrated Insect Immunology: From Basic Biology to Environmental Applications, Pultusk, Poland 23-28 September 2013.

Pike, A., S. Sandiford, A. Vadlamani and G. Dimopoulos. "The Rel2 regulated transcriptome and proteome of Rel2 overexpressing *Anopheles stephensi*," poster presented as part of the Keystone Symposia on Molecular and Cellular Biology: Malaria, New Orleans, LA, 20-25 January 2013.

Pike, A., S. Sandiford, A. Vadlamani and G. Dimopoulos. "The Rel2 regulated proteome of the malaria vector *Anopheles stephensi*," poster presented as part of the 2012 Summer Frontiers Symposium on "Training the innate immunity: immunological memory in innate host defense," Nijmegen, The Netherlands, 28-29 June 2012.

Pike, A., G. Bian, Y. Xu, and Z. Xi. "Effects of *Wolbachia* and dengue virus infection on the mosquito *Aedes aegypti,*" poster presented as part of the 2009 Entomological Society of America Annual Meeting, Indianapolis, IN, 13-16 December 2009.

Pike, A., G. Bian, Y. Xu, and Z. Xi. "Role of innate immunity in regulation of *Wolbachia* infection level in *Aedes aegypti,*" poster presented as part of the 2009 American Society of Tropical Medicine and Hygiene Annual Meeting, Washington DC, 18-22 November 2009.

GRANTS and FELLOWSHIPS

Tropical Medicine Dinner Club of Baltimore Simpson Student Award in Tropical Disease Field Research, April 2014: $500 to defray costs for travel or research in a developing country

Johns Hopkins Center for Global Health Field Research Award, March 2014: $3500 to travel to an international site for at least 4 weeks of field research

Johns Hopkins Malaria Research Institute pre-Doctoral Fellowship, Jan. 2013-Dec. 2014: Two years of health insurance and fellowship support for pre-doctoral fellows interested in malaria research

Michigan State University Department of Entomology Hutson Travel Fellowship Feb. 2010: $400 to travel to the national meetings of the Entomology Society of America Annual Meeting

RESEARCH EXPERIENCE

Graduate Student: Ph.D. – Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD. Rotations in the George Dimopoulos, Fernando Pineda and Jason Rasgon labs working on *Anopheles gambiae* mosquito immunity, modeling mitochondrial movement in neurons, and the introduction of *Wolbachia* into *Anopheles* spp. mosquitoes, respectively. Currently working in the laboratory of Dr. George Dimopoulos on the effects of transgenic immune deployment on mosquito fitness and gene expression levels.

Graduate Student: M.S. – Michigan State University, East Lansing, MI. Working in the laboratory of Dr. Zhiyong Xi on the three way interactions between dengue virus, its mosquito host, and the intracellular endosymbiont bacterium *Wolbachia*.

Undergraduate Student – Oberlin College, Oberlin, OH. Working in the laboratory of Dr. Mary Garvin looking for evidence of vertical transmission of West Nile Virus in the mosquito *Culiseta melanura* as a possible overwintering mechanism for West Nile Virus in temperate climates.

OTHER EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Teaching Assistant. January 2013-Present.

Michigan State University Integrative Studies Biological Program, Teaching Assistant. August 2009-May 2010.

Oberlin College Student Academic Services, Peer Liaison. January 2006 – May 2008.

Oberlin College Department of Computer Science, TA. September 2007 – May 2008.

Oberlin College Department of Mathematics, tutor and grader. September 2004 - May 2007.

MEMBERSHIPS

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