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THREE WAY INTERACTIONS BETWEEN *WOLBACHIA*, DENGUE VIRUS, AND THEIR HOST, *AEDES AEGYPTI*

presented by

ANDREW PIKE

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Entomology

Major Professor's Signature

06/02/2010

Date

THREE WAY INTERACTIONS BETWEEN *WOLBACHIA*, DENGUE VIRUS, AND THEIR HOST, *AEDES AEGYPTI*

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Entomology

2010

ABSTRACT

THREE WAY INTERACTIONS BETWEEN *WOLBACHIA*, DENGUE VIRUS, AND THEIR HOST, *AEDES AEGYPTI*

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Many vector-borne diseases, including dengue virus, have no vaccines or specific treatments, leaving vector control as the main tool to fight them. The intracellular bacterium *Wolbachia* has been proposed as a potential control agent for vector mosquitoes, including *Aedes aegypti*. Before *Wolbachia* can be used as a vector control tool, we must better understand the interactions between the bacterium, its host, and the pathogen it targets. We used a variety of techniques to activate or suppress the mosquito immune system, and measured the change in *Wolbachia* densities in both mosquito ovaries and carcasses using real-time quantitative PCR (qPCR). Simultaneously inducing both the Toll and Imd pathways leads to an increase in ovary *Wolbachia* densities while repressing the Imd pathway or infection with gram negative bacteria decreases *Wolbachia* densities in mosquito ovaries. No effects on *Wolbachia* densities in mosquito carcasses were observed. We also measured how infection by *Wolbachia*, dengue virus, or both affects the fitness of the mosquito host. Co-infected mosquitoes live longer than mosquitoes infected with only the virus. Neither virus nor *Wolbachia* infection affected the number of eggs laid or the egg hatch rate. We conclude that the mosquito immune system is can affect *Wolbachia* infection levels and *Wolbachia* infection can have a positive effect on mosquito life history, especially when the mosquito is also infected with dengue virus.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Zhiyong Xi, for allowing me to work with him on this project. Similarly, I would like to thank the other members of my graduate committee, Drs. Jim Miller and Ned Walker, for their advice and guidance. I also greatly appreciate the help and advice provided in by Dr. Guowu Bian and Yao Xu in the lab. I would next like to thank Jeff Landgraf and the rest of the staff of the MSU genomics core for helping me run a lot of real time qPCR. Thank you also to the entire staff of the Entomology Department; Heather Lenartson-Kluge, Carolyn Lewis, Linda Gallagher, Chrissi Smith, Jan Eschbach, and Brooke Gallagher, for helping me in many ways and keeping me on track to graduate. None of this would have been possible without the support of our department chair, Dr. Ernest Delfosse, to whom I am greatly indebted. Finally, thank you to my family and friends for all their advice, editing, and emotional support while I was writing this thesis.

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CHAPTER 1

Interactions among vectors, symbionts, and pathogens

Introduction

Mosquitoes are among the most important insect pests in the world. Many significant human pathogens are spread by mosquitoes, including the viruses causing West Nile Encephalitis, Dengue fever and chikungunya fever, malaria parasites, filarial nematodes, and many others. These parasites have adapted themselves to the vector-borne life cycle by taking advantage of the female mosquitoes' need to take a vertebrate blood meal before laying eggs. While utilizing a two-host system allows the disease-causing agents access to new hosts, the pathogens are presented with a unique set of challenges in avoiding two distinct immune systems. Despite these challenges many diseases are spread in this way, and cause an overwhelming amount of human morbidity and mortality each year.

One important vector species is the yellow fever mosquito, *Aedes aegypti*. Originally from Africa, this species of mosquito, which is the main vector for yellow fever virus and dengue virus, has spread around the world and is now found in tropical areas worldwide (Mousson et al. 2005). This range expansion of *Ae. aegypti* has led to a parallel expansion of the diseases it carries, including dengue fever (Vezzani and Carbajo 2008). Along with physiological adaptations that make it a good disease vector, this mosquito species has adapted itself to an urban lifestyle, with behaviors such as laying eggs in man-made water containers and rarely straying far from human habitation. This close association with people

increases the likelihood that it will bite humans as well as its vectorial capacity. Due to its importance as a vector of human disease, considerable attention has been paid to this mosquito in the scientific community, including the sequencing of its genome and the creation of transgenic techniques. However, it remains a serious threat to people (Adelman et al. 2002, Nene et al. 2007).

Dengue virus, the causative agent of dengue fever and the associated dengue hemorrhagic fever, is the most important arbovirus in the world. Approximately 50 million cases of infection are reported each year, and 2.5 billion people are at risk throughout the world today (WHO 2009). Dengue virus is unique among vector-borne diseases in that it is maintained completely in an urban cycle, transmitted only between humans and mosquitoes, with no need for input from an animal reservoir (Wang et al. 2000). Because there is no effective vaccine for dengue virus, people rely on vector control strategies to prevent the disease. Historically, these control strategies have included pesticide sprays, removal of mosquito habitats, and biological control; however, these have not proven effective enough to be considered long term control systems (Kyle and Harris 2008). Due to the lack of good vector control mechanisms, the range and severity of dengue outbreaks continue to increase and new control strategies are under investigation.

Wolbachia pipientis, first described in the mosquito *Culex pipiens*, is an intracellular alphaproteobacteria that infects approximately 66% of insect species (Yen and Barr 1971, Jeyaprakash and Hoy 2000, Hilgenboecker et al. 2008). Generally classified as a reproductive parasite, *Wolbachia* is able to manipulate

its host's reproduction in various ways, including male-killing, parthenogenesis induction, feminization of genetic males, and cytoplasmic incompatibility (CI), in order to facilitate its spread into a host population (Werren 1997, Werren et al. 2008). In some host species, *Wolbachia* can have other functions, including serving as a nutritional symbiont in bedbugs (Hosokawa et al. 2010).

CI has been proposed as a vector control mechanism, either as a drive mechanism to replace the current vector population with one bearing genes reducing vectorial capacity or as a population suppressant (Dobson et al. 2002, Sinkins and Godfray 2004, Sinkins and Gould 2006). *Wolbachia* population replacement has been seen not only in laboratory insect populations, but also in natural *Drosophila* populations in California (Turelli and Hoffmann 1991). To facilitate the usefulness of *Wolbachia* for vector control, researchers have recently developed techniques to transfer *Wolbachia* strains between hosts, allowing them to infect previously uninfected species such as *Ae. aegypti* (Xi et al. 2005, McMeniman et al. 2009). One particular strain, *w*MelPop, shortens the lifespan of both *Drosophila* and *Ae. aegypti*, suggesting another *Wolbachia*-based vector control strategy: changing the host population structure so that mosquitoes do not survive through the extrinsic incubation period of the parasite, and thus never become infective (Min and Benzer 1997, McMeniman et al. 2009).

While considerable research has been performed on each of the individual organisms listed above, there is still a lack of knowledge about the interactions between them. Numerous researchers have investigated two-way interactions

between mosquitoes and pathogens or symbionts, but little has been performed looking into how three-way infections change the infection dynamics. Figure 1.1 shows a diagram of possible two-way interactions between a mosquito host, *Wolbachia,* and a generalized pathogen. If *Wolbachia* is to be employed as a control mechanism for *Ae. aegypti*, the vector of dengue virus, it is important to understand the ways that infection by both the symbiont and the pathogen will affect the mosquito host and each of the microorganisms. Therefore, investigations into the ways that infection dynamics change during co-infection deserve further study. Here, I review previous research and present suggestions for future directions.

Three way interactions

Effects of endosymbiont infection on pathogens

Recent evidence has shown that *Wolbachia* infected insects have increased resistance to various viruses, including those causing human disease (Hedges et al. 2008, Teixeira et al. 2008, Moreira et al. 2009b, Bian et al. 2010). In a series of experiments on *Ae. aegypti* artificially infected with *w*MelPop, Moreira et al. (2009) showed that fewer mosquitoes infected with *Wolbachia* became infected with dengue virus after an infectious blood meal than either those cured of the *Wolbachia* infection with tetracycline or naturally uninfected mosquitoes. Those mosquitoes that did develop a dengue infection also had fewer copies of the virus in their systems, and immunofluorescence stains showed that *Wolbachia* and dengue virus do not inhabit the same mosquito cells.

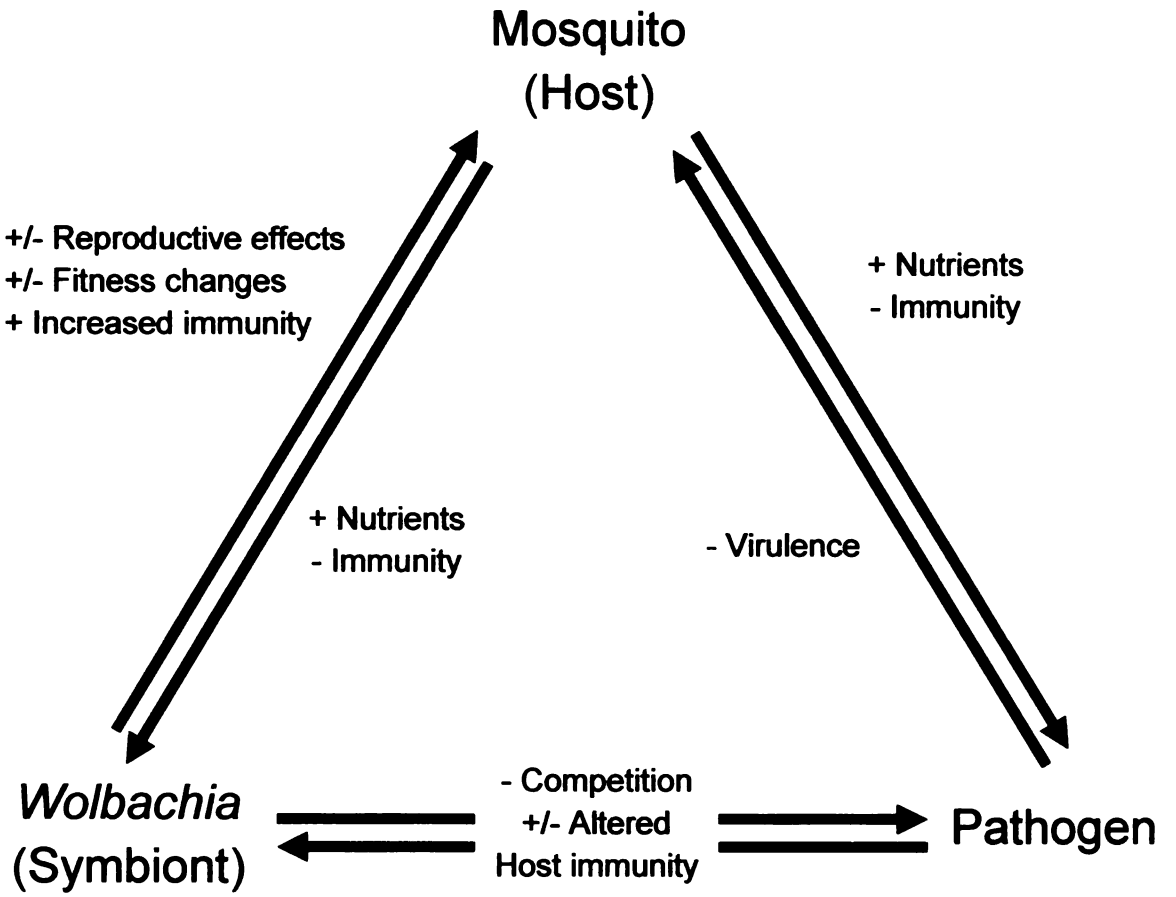

Figure 1.1: Illustration of possible interactions between mosquitoes, *Wolbachia* and vector-borne pathogens. + indicates positive interactions, while - indicates negative interactions.

Ae. aegypti mosquitoes infected with *Wolbachia* also developed lower titers of Chikungunya virus and had fewer *Plasmodium gallinaceum* oocysts develop in their midguts than mosquitoes with no *Wolbachia* infection. However, the effects of *Wolbachia* infection on *P. gallinaceum* were not observed in the naturally *Wolbachia* infected mosquito *Ae. fluviatillis*. Bian et al. (2010) also saw that another artificially *Wolbachia* infected line of *Ae. aegypti* had increased resistance to dengue virus infection. Mosquitoes infected with the *w*AlbB strain of *Wolbachia* developed significantly lower dengue virus titers than those lacking *Wolbachia* infection, signifying again that *Wolbachia* interferes with dengue virus replication and spread in mosquitoes.

These results hold for insects other than mosquitoes as well. Heges et al. (2008) found that *Drosophila melanogaster* infected with either the *w*MelCS or *w*MelPop strain of *Wolbachia* lived significantly longer after infection by *Drosophila* C virus than flies cured of *Wolbachia* infection by antibiotic treatment. This increase in longevity correlated with delayed accumulation of virus in the *Wolbachia* infected flies. In the same study, the authors found that *Wolbachia* infected flies co-infected with either Cricket Paralysis virus or Flock House virus also lived longer than *Wolbachia*-free conspecifics. In an independent study, Teixeira et al. (2008) found that *D. melanogaster* infected with *Wolbachia* had increased resistance to *Drosophila* C virus, Nora virus, and Flock House virus. Flies infected with *Wolbachia* had longer lifespans after infection with the viruses. This effect was related to a lack of virus proliferation in fly tissues. However, *Drosophila* resistance to Insect Iridescent Virus 6 did not differ significantly

between *Wolbachia*-infected and uninfected flies. Because *Drosophila* C virus and dengue virus are both single-stranded positive-sense RNA viruses, while Insect Iridescent Virus 6 is a DNA virus, these results support the evidence that interactions between *Wolbachia* and dengue virus when the two co-infect a mosquito host could reduce the global dengue burden.

Wolbachia is also able to impact the spread of pathogens by reducing the lifespan of mosquitoes it infects. While some strains of *Wolbachia* increase the lifespan of mosquitoes, the *w*MelPop strain of *Wolbachia* reduces the lifespan of both its natural host, *D. melanogaster*, and artificially infected *Ae. aegypti* (Dobson et al. 2004, Calvitti et al. 2009, McMeniman et al. 2009). Due to the extrinsic incubation period of vector-borne diseases, shortening the vector lifespan can greatly reduce pathogen transmission rates by simply causing the mosquitoes to die before they become infective. However, this effect is specific to the *w*MelPop variant of *Wolbachia*. In *Ae. albopictus*, infection with the naturally occurring *w*AlbA and *w*AlbB strains of *Wolbachia* actually increases the lifespan of female mosquitoes, though there was no such effect on males (Dobson et al. 2004, Calvitti et al. 2009). Similarly, when co-infected with dengue, *w*AlbB increases the lifespan of artificially infected *Ae. aegypti* (Bian et al. 2010). This fact makes *w*MelPop seem more like a mosquito pathogen than *w*AlbB (Suh et al. 2009).

Wolbachia infection also affects host fitness in a variety of other ways which can increase or decrease the spread of vector-borne diseases. *w*MelPop infection of *Ae. aegypti* increases the activity of the mosquitoes, which could lead

to increased biting rates (Evans et al. 2009). If the greater locomotory activity translates to more human contact, this increased activity could lead to more infective bites, and could actually increase the mosquito's vectorial capacity, despite decreasing their lifespan. Counter to this, however, is the observation that *w*MelPop-infected mosquitoes are less able to successfully take blood meals (Moreira et al. 2009a, Turley et al. 2009). *Wolbachia* infected mosquitoes were shown to take longer before biting, complete fewer successful bites, and have shaky or bendy proboscides; these effects were age-dependent. Therefore, in general, the *w*MelPop infected mosquitoes could not take blood meals as well as their uninfected conspecifics, and the effect became more pronounced as the mosquitoes aged. Even if the *Wolbachia* infected mosquitoes become more active and attempt to bite humans more often, if they are more likely to fail to feed successfully or have their feeding attempts end in death, these effects can combine to greatly reduce their vectorial capacity.

The above symbiont-mediated pathogen resistance can occur in a variety of ways. First, symbionts are also able to protect their hosts from a variety of pathogens other than viruses. The life-shortening *w*MelPop strain of *Wolbachia* has also been shown to reduce the number of surviving filarial worms in *Ae. aegypti* (Kambris et al. 2009). Similar to the effects of *Wolbachia* on virus infection levels, *w*MelPop-infected mosquitoes have significantly fewer third stage larvae of *Brugia pahangi*, indicating that the protective effects of *Wolbachia* extend beyond viruses and into multicellular worms. In *D. melanogaster*, *Wolbachia*-infected female flies are more resistant to a fungal pathogen, while

Wolbachia-infected males have a competitive advantage over uninfected males when infected with a bacterial pathogen (Panteleev et al. 2007). Second, symbionts other than *Wolbachia* can also induce resistance to numerous pathogens. In the pea aphid *Acyrthosiphon pisum*, the vertically transmitted bacteria *Regiella insecticola* confers resistance to the common fungal pathogen *Pandora neophidis*. In experiments on five different aphid clone lines, significantly more aphids survived fungal infection when infected with *Regiella* than when uninfected by the bacteria, and similarly, fungus on aphids bearing *Regiella* infection was significantly less likely to sporulate than fungus on aphids without *Regiella* (Ferrari et al. 2004, Scarborough et al. 2005). These results show that the symbionts are able to protect not only their host, but also nearby conspecifics by reducing the ability of a pathogen to spread after infection. These effects are even noticeable in humans, where infection with scrub typhus caused by the bacteria *Orientia tsutsugamushi*, a close relative of *Wolbachia,* causes reduced infection with HIV (Watt et al. 2000).

Some studies on other organisms point to the opposite effect: that infection with endosymbionts can actually increase vectorial capacity of insects. For instance, in order for the whitefly *Bemisia tabaci* to successfully spread Tomato yellow leaf curl virus, the insect must be infected with the vertically transmitted symbiont *Buchnera*, which increases the hemolymph levels of a homologue of the molecular chaperone GroEL (Morin et al. 1999, Morin et al. 2000). When the GroEL homologue is removed by treatment with anti-*Buchnera* GroEL antisera, transmission of Tomato yellow leaf curl virus is reduced by more

than 80%, indicating that interactions between the chaperone and virus is interrupted, disrupting a necessary step of transmission. Similar results were seen in *Myzus persicae* aphids spreading Potato leaf roll virus (van den Heuvel et al. 1994). When the bacterial symbionts of the aphid were removed by antibiotic treatment, levels of the protein symbionin were reduced in the hemocoel. This reduction was matched by a reduction in the transmission of Potato leaf roll virus. Interestingly, *Wolbachia* is also able to make some hosts more susceptible to pathogens. *Wolbachia* infected *Drosophila simulans* exhibited reduced ability to encapsulate eggs of the parasitoid wasp *Leptopilina heterotoma*, though there was no reduction in ability to survive fungal infections (Fytrou et al. 2006). This effect is likely due to the ability of *Wolbachia* to reduce the cellular immune response of the flies.

Effects of pathogen infection on symbionts

When acting as the sole infection of an insect, symbionts must maintain a delicate balance of infection level; infection level must be high enough to guarantee transmission to the next generation, and yet be low enough not to injure the host in a significant way (Werren 1997). However, co-infection of the host with both a symbiont and a pathogen can disrupt this balance. The density of *Wolbachia* infection has been shown to depend on a variety of host life factors including: density of larvae, age of the insect host, and presence or absence of insecticide resistance genes (Berticat et al. 2002, McGraw et al. 2002, Wiwatanaratanabutr and Kittayapong 2009). These variations in *Wolbachia*

density are seen not only in the lab, but also in natural populations (Ahantarig et al. 2008b, Ahantarig et al. 2008a). In *Drosophila* and other insects, the density of *Wolbachia* infection correlates directly to the strength of cytoplasmic incompatibility observed, indicating that variation in infection levels can lead to decreased efficacy of *Wolbachia* as a gene drive mechanism (Breeuwer and Werren 1993, Sinkins et al. 1995, Perrot-Minnot et al. 1996, Unckless et al. 2009). However, evidence from *Culex pipiens* suggests that the density-dependent strength of CI may not generalize to all insects. It remains to be seen how much, if at all, the *Wolbachia* density affects the strength of CI in important vector species such as *Ae. aegypti* (Duron et al. 2009).

Another important factor affecting the density of *Wolbachia* in an insect host is infection with other microbes (Duron et al. 2008). *Ae. albopictus* mosquitoes infected with both *Wolbachia* and Chikungunya virus, a member of the Togaviridae virus family, had lower *Wolbachia* densities than mosquitoes lacking Chikungunya infection, possibly indicating that co-infection of *Wolbachia* and a viral pathogen in the same host can lead to decreased *Wolbachia* levels, or possibly even the loss of *Wolbachia* infection (Tortosa et al. 2008). Conversely, it has also been observed that co-infection with a pathogen and *Wolbachia* can lead to an increase of *Wolbachia* in the host (Rio et al. 2006). Rio et al. (2006) showed that co-infection of male tsetse flies with *Wolbachia* and trypanosomes leads to an increase in the *Wolbachia* density, indicating that the dual infection has a positive effect on the symbiont. However, these results

involve a protozoan parasite, not a virus, and thus may not be applicable to the dengue virus-*Wolbachia* interactions in mosquitoes.

If infection with a pathogen were able to impact the *Wolbachia* infection level of their host, it could, over time, lead to the complete removal of *Wolbachia* from the host. Horizontal gene transfer from *Wolbachia* to insects has been documented in numerous insect species, including species that do not contain *Wolbachia* in nature such as *Ae. aegypti* and *Anopheles gambiae*, indicating that at some point in the past these species were infected with *Wolbachia* and have since lost the infection (Hotopp et al. 2007, Woolfit et al. 2009). One possible explanation for the loss of infection is interspecific competition between the *Wolbachia* and other microbes, which would explain the lack of *Wolbachia* infection in such vector species as *Ae. aegypti*, *An. gambiae*, and *Culex tritaeniorhynchus* (Kittayapong et al. 2000, Tsai et al. 2004).

Effects of dual infection on the host

Infection by pathogens and endosymbionts is not without cost to the mosquito host. *Wolbachia* has obvious effects on the host reproductive biology (Werren 1997). Dengue infection alone has been shown to decrease both mosquito lifespan and feeding efficiency, though the results have not been consistent across all studies (Putnam and Scott 1995, Platt et al. 1997, Joshi et al. 2002, Hanley et al. 2008). Platt et al. (1997) showed that dengue-infected mosquitoes took longer to take a blood meal than uninfected mosquitoes, which could be associated with increased mortality, but would also increase the

probability that the mosquitoes obtain or pass on dengue virus. Joshi et al. (2000) found that mosquitoes infected with dengue virus by intrathoracic injection had higher mortality than those injected with only media. In addition, the larval stages lasted longer for progeny of infected females. On the other hand, Putnam and Scott (1995) saw no significant change in mosquito feeding behavior after infection with dengue virus, and Hanley et al. (2008) did not observe a difference in survival between dengue-infected and uninfected *Ae. aegypti*. We have observed that dengue virus decreases the longevity of *Ae. aegypti* when infected with an artificial blood meal, but co-infection of *Wolbachia* with dengue virus removes at least the lifespan effects, indicating that being infected by both the symbiont and the pathogen can be advantageous for the host (Bian et al. 2010).

The full effects of dual infection on the host have yet to be elucidated; however, it has been theorized that such a co-infection may lead to either increased or decreased virulence of the pathogens (Frank 1994, Baalen and Sabelis 1995, Frank 1996). One school of thought posits that co-infection by multiple unrelated parasites can increase virulence because more virulent exploiters of host resources will have a competitive advantage over those that take more time (Hardin 1968). However, other researchers have conjectured that the presence of multiple competing parasites can reduce the ability for any one to pull ahead, which would reduce the virulence of each by underuse of host factors (Chao et al. 2000). While the mechanism of the relations and the effects of dual-infection upon the host remain unclear, either result will be different from the effects of single infections alone.

Mechanisms

Two main mechanisms have been proposed to explain the interactions between pathogens, symbionts, and hosts: competition and host immunity. Generally speaking, when two organisms compete for the same limited resource, they cannot co-exist and one will become extinct (Hardin 1960). This could be happening in the case of symbiont-pathogen interactions within insect hosts. Moreira et al. (2009) found that *Ae. aegypti* cells are infected by only *Wolbachia* or dengue virus, not both. This localized exclusion suggests that individual cells are colonized by either the symbiont or the pathogen, and that microbe utilizes all of the available resources, keeping the other from invading. *Ae. albopictus* cells grown in culture and infected with Sindbis virus were seen to limit superinfection with closely related viruses by releasing antiviral agents (Condreay and Brown 1986). Similarly, it has been shown that individual ticks are not infected with multiple *Rickettsia* spp. at the same time, even when the tick species is known to vector multiple rickettsiae, implying that competition between the pathogens restricts ticks to carrying only one at a time (Azad and Beard 1998). If these results generalize to interactions between more distantly related microbes, such as *Wolbachia* and dengue virus, they could explain why *Wolbachia*-infected mosquitoes act as inferior vectors for dengue, and why the most competent vectors lack *Wolbachia* infections.

Activation of the insect immune system could also limit infection of the host to one microbe at a time. While mosquitoes, like all insects, lack the

adaptive immune system found in mammals, they are still able to react to invaders with an innate immune system (Hoffmann 1995). Two main pathways, the Toll and Immune deficiency (Imd) pathways, are responsible for much of the immune response of insects to infection and have been well studied in *Drosophila* and mosquitoes. Two other, less well-studied, pathways, the RNA interference and JAK-STAT pathways, also play a role in immunity (Hoffmann et al. 1996, De Gregorio et al. 2002, Hoffmann 2003, Sanchez-Vargas et al. 2004, Dostert et al. 2005). Generally, the Toll pathway is activated in response to infection with Gram-positive bacteria, viruses, and fungi, while the Imd pathway reacts to invasion by Gram-negative bacteria, though there is interplay between the two. Though initial results indicated that *Wolbachia* infection has no effect on the expression of insect immune effector genes, recent studies have shown that, in fact, *Wolbachia* infection can lead to a significant increase in the levels of numerous important insect immunity genes (Bourtzis et al. 2000, Xi et al. 2008b)

In *Drosophila*, the Toll pathway was activated in response to infection by both *E. coli* and the *Drosophila* X virus, leading to the release of the same immune effectors (Zambon et al. 2005). Similarly, in mosquitoes, it has been shown that up-regulation of immune pathways can lead to reduced infection by dengue virus, nematodes, and protozoans, and in tsetse flies the immune system regulates the infection dynamics of trypanosomes (Hu and Aksoy 2006, Xi et al. 2008a, Garver et al. 2009, Kambris et al. 2009, Sanchez-Vargas et al. 2009). It is possible that one microorganism could cause the up-regulation of the host immune system, thereby removing other microorganisms from the host while

avoiding the immune effects itself. Studies have shown that, in mosquitoes, removal of endogenous microbial flora can lead to reduced immune activation and increased susceptibility to malaria parasites and dengue virus, and also that numerous immune genes are down-regulated after the microflora is removed. (Xi et al. 2008a, Dong et al. 2009) In tsetse flies, removal of the nutritional symbiont *Wigglesworthia glossinidia* by antibiotic treatment led to greater trypanosome infections; the authors conjecture that the reason for this effect is reduced immunity (Pais et al. 2008). Both of these results suggest that the microbes infecting a host can affect the immune system of the host enough to reduce infection by a second microorganism.

A third explanation for the observed interactions between symbionts and pathogens in the same host is also possible: toxic compounds may be released by one microbe that affect the other or the host. Many microbial organisms are capable of releasing toxic compounds for protection or pathogenicity. It is possible that *Wolbachia* releases a compound that damages dengue virus particles or dengue does the same to *Wolbachia*. There are examples of endosymbiotic bacteria providing protection for their hosts in just this way. For instance, *Alteromonas* spp. bacteria inhabiting the shrimp *Palaemon macrodactylus* release the anti-fungal compound isatin, thereby protecting the shrimp from the fungus *Lagenidium callinectes* (Gil-Turnes et al. 1989). This may also be the mode of action by which the bacteria *Regiella insecticola* acts to protect its pea aphid host, though no anti-fungal compounds released by *Regiella* have yet been identified (Ferrari et al. 2004, Scarborough et al. 2005). However,

no such compounds have yet been identified in either *Wolbachia* or dengue virus. Also, the fact that *Wolbachia* and dengue are not found to occupy the same mosquito cells and that the bacteria can induce an immune response indicate that competition or immune response may be the actual mechanism (Moreira et al. 2009b, Bian et al. 2010).

Evolutionary implications

The interactions between parasites and their hosts can have many effects on the evolution of all involved organisms; these effects may vary when multiple infection types are present. When *Wolbachia* alone infects mosquitoes and causes CI, it creates an evolutionary tension between males and females. Because infected females have an advantage over uninfected females, they should select for genes that increase the likelihood of their infection and the likelihood that they pass the infection on to their offspring (Turelli 1994). However, because infected males cannot successfully mate with uninfected females, being infected has a cost for males when *Wolbachia* infection is not fixed in the population, implying that low infections may be favored among males. These two selective forces would then act in opposition to each other. These exact predictions have been shown in *Aedes albopictus* infection by *w*AlbA type *Wolbachia*; *w*AlbA infections in females increase over time while the decrease in males (Tortosa et al. 2010).

In addition, there is evolutionary pressure on any symbiont or parasite to evolve mechanisms to avoid host immune responses so that they are not

removed from the system. Counter to this is selection by the host to increase or maintain immune response in order to fight off infection and survive. Also, many parasites alter host behavior to increase the likelihood of spreading the infection, including changes in feeding behavior and life history traits, which could, over time, lead to permanent changes in the host (Hurd 2003).

These evolutionary dynamics only become more complex when multiple infections affect the same host. When multiple microbes are utilizing host resources, the effects on the host may be increased. This would lead to increased selective pressure on the host to evolve methods to limit the infection level of the parasites. At the same time, the two parasites would compete for limited host resources which could affect the evolution of virulence. It is possible that one would evolve to be more virulent so it could quickly outcompete the other microbe, possibly removing it completely from the host system (Hardin 1960). Conversely, it is possible that both microbes could evolve towards reduced virulence so that the host does not die and both microbes could occupy the same host without either being able to use all of the host resources (Chao et al. 2000). This second situation has been seen many times in nature, such as the multiple *Wolbachia* infection of many insects including *Aedes albopictus* and the infection of tsetse flies by three symbionts (Dobson et al. 2001, Rio et al. 2006). If the first theory is correct and co-infection by a symbiont and a pathogen leads to increased virulence of the pathogen, it could increase the transmission of the disease and have wide-ranging implications for disease control efforts.

This thesis reports on research into the specific interactions between *Ae. aegypti, Wolbachia*, and dengue virus type-2. Chapter 2 reports on laboratory experiments testing the effects of the mosquito immune system on *Wolbachia* densities in the mosquito host. Chapter 3 describes new information on the effects of co-infection by both *Wolbachia* and dengue virus type-2 on mosquito lifespan and fecundity. Finally, the concluding chapter summarizes these results and discusses their implications for future research and disease control.

CHAPTER 2

Effects of the mosquito immune system on *Wolbachia* density

Introduction

Originally from Africa, the yellow fever mosquito *Aedes aegypti* has spread to tropical areas around the world, and is a species of great concern due to its status as the main vector of dengue virus (Mousson et al. 2005). Dengue virus, the causative agent of dengue fever and the deadly dengue hemorrhagic fever, is the most important arbovirus currently circulating, causing 50-100 million cases of human disease each year (WHO 2009). Despite the severity of dengue and the large amount of research effort invested in it, there is not yet an effective vaccine or specific treatment for this disease. This leaves vector control strategies as the main disease control mechanism. Past attempts to control the mosquitoes that spread dengue used traditional methods, such as chemical insecticides and cultural practices like removing breeding grounds. Despite early successes, these methods have not provided a long-term solution to the dengue problem (Kyle and Harris 2008). Thus, recent efforts have looked to modification of the vector populations by genetic engineering or population suppression to reduce dengue transmission in place of killing off the mosquitoes. One proposed method of mosquito population control is dependent upon infection by the endosymbiotic bacteria *Wolbachia pipientis* (Engelstadter and Telschow 2009).

Wolbachia, maternally transmitted alphaproteobacteria, cause a variety of reproductive modifications in their arthropod hosts, including male killing, feminization of genetic males, induction of parthenogenesis, and cytoplasmic

incompatibility (CI) (Werren et al. 2008). Because *Wolbachia* infect approximately 2/3 of insect species, as well as numerous other arthropods, and are able to spread quickly through populations due to their reproductive modifications, they have recieved a great deal of attention as potential vector control tools (Hilgenboecker et al. 2008). CI, a condition in which mating crosses between infected males and uninfected females lead to karyogamy failure and death of embryos while embryos from all other crosses develop normally, has been specifically identified as having great potential for reducing vector-borne disease burden (Werren et al. 2008). Because through CI *Wolbachia* halts successful matings between infected males and uninfected females, while infected females are able to mate with all males, infected females are provided a reproductive advantage over their uninfected counterparts. This causes *Wolbachia* to spread quickly into a population, which has been observed both in the laboratory and nature (Turelli and Hoffmann 1991, Xi et al. 2005). If *Wolbachia* are linked to a gene of interest, they can drive that gene into the population. This could be accomplished by genetically modifying the *Wolbachia* or another co-infecting maternal transmitted microbe for paratransgenesis (Olson et al. 1996, Turelli and Hoffmann 1999, Franz et al. 2006).

Recent studies have also indicated that *Wolbachia* infection alone can confer resistance to a variety of viruses, increasing its possible usefulness for vector-borne disease control. Studies in mosquitoes have shown that *Ae. aegypti* artificially infected with either the *w*AlbB or *w*MelPop strains of *Wolbachia* also develop lower titers of dengue virus, chikungunya virus and *Plasmodium*

gallinaceum, and have fewer adverse health effects than *Wolbachia* uninfected mosquitoes (Moreira et al. 2009b, Bian et al. 2010). Many of the *Wolbachia*-infected mosquitoes effectively cleared viruses from their system, rendering them ineffective as disease vectors. In addition to limiting the replication levels of various parasites, some strains of *Wolbachia* have also been shown to shorten their host lifespans, both in naturally and artificially infected hosts (Min and Benzer 1997, McGraw et al. 2002, McMeniman et al. 2009). This may increase the bacteria's usefulness for disease control, as they could kill off hosts before the extrinsic incubation period of the disease-causing agent passes, potentially stopping or reducing transmission. However, these lifespan-reducing effects are not universal to *Wolbachia* strains, as some have no effect on host lifespan, and some even increase it (Dobson et al. 2004, Xi et al. 2005, Calvitti et al. 2009). Also, in *Drosophila melanogaster*, *Wolbachia* infection increased the lifespan of flies infected with *Drosophila* C virus and decreased the virus infection level (Hedges et al. 2008, Teixeira et al. 2008). Similar results were also seen in flies infected with Flock House virus, Cricket Paralysis virus and Nora virus, with *Wolbachia* infection reducing the phenotypic effects of virus infection.

Before *Wolbachia* can be used in any capacity to control vector-borne disease, the interactions between the mosquitoes, the bacterium and any pathogens we wish to control must be better understood. The density of *Wolbachia* infecting insect cells may affect the strength of CI, and thus the effectiveness of a vector control program, so if infection with a pathogen reduces

Wolbachia levels it may also decrease our ability to employ *Wolbachia* for our own benefit (Sinkins et al. 1995, Duron et al. 2009).

Although *Ae. aegypti* mosquitoes are not naturally infected with *Wolbachia*, they are able to be infected and act as an amenable host to the bacteria (Xi et al. 2005, McMeniman et al. 2009). There is evidence for an ancient horizontal gene transfer between *Wolbachia* and mosquitoes, including *Ae. aegypti* and *Anopheles gambiae*, though the direction of the transfer is unknown (Woolfit et al. 2009). Also, many close relatives of *Ae. aegypti*, including *Ae. albopictus*, are infected with *Wolbachia*. The fact that *Ae. aegypti* act as good hosts to *Wolbachia*, bear *Wolbachia* genes, and have *Wolbachia* infected relatives indicates that at one time in their evolutionary history they were infected with *Wolbachia* and have subsequently lost the infection. The reason for this loss is unknown, but possible reasons include competition with other microbes and interactions with the mosquito immune system.

Mosquitoes, like all insects, have only an innate immune system; they lack the adaptive immune system found in mammals (Hoffmann 1995). However, the insect immune system is able to mediate the effects of many infections. The Toll and Imd pathways are the best-studied pathways, but the RNA interference and JAK-STAT pathways also play an important role in insect immunity (Hoffmann et al. 1996, De Gregorio et al. 2002, Hoffmann 2003, Sanchez-Vargas et al. 2004, Dostert et al. 2005). The Toll pathway, which reacts specifically to Gram-positive bacteria, viruses, and fungi, is responsible for mediating dengue virus infection, while the Imd pathway reacts to Gram-negative bacteria (Xi et al. 2008a). In

mosquitoes, the Toll pathway is activated when its negative regulator, *Cactus*, releases from the transcription factor *Rel1*, allowing it to localize to the nucleus and induce transcription of numerous antimicrobial peptides. Similarly, the Imd pathway works when its negative regulator, *Caspar*, dissociates from *Rel2*, again causing nuclear localization and increased expression of antimicrobial peptides. These peptides are then released into the cytoplasm or intracellular matrix where they act to fight off any invading pathogens.

Previous studies have shown that the mosquito immune system can be altered by RNA interference, genetic modification, and the addition or removal of microbial flora (Xi et al. 2008a, Antonova et al. 2009). Here, we use these well developed methods to study the role mosquito immunity plays in modulation of *Wolbachia* density. Our results indicate that the mosquito immune system may indirectly, but not directly, influence *Wolbachia* densities in *Ae. aegypti* ovaries.

Materials and Methods

Mosquito Rearing. Ae. aegypti mosquitoes of the artificially *Wolbachia*-infected WB1 strain were reared according to standard procedures. Eggs were hatched in double distilled water under a vacuum, and larvae were reared at low densities (approximately 200 larvae/pan) in plastic pans containing 750 mL of distilled water. Larvae were allowed to feed constantly on a 6 g/100 mL liver powder solution in water until pupation. Pupae were moved to a cup of distilled water in a cage where they eclosed and adults lived. Adults were constantly provided a

10% sucrose solution in cotton balls and kept at 27° C and 80% humidity on a 12:12 hour light/dark cycle.

Real-time quantitative polymerase chain reaction (qPCR): Real-time qPCR was used to measure the number of copies of the *Wolbachia* single copy *wsp* gene using a standard curve. The number of copies of the *Wolbachia* genome was normalized against the mosquito ribosomal protein S6 (*RPS6*) gene. Mosquitoes were anesthetized with CO_2; their ovaries were dissected out in sterile STE buffer and heads were removed. During RNAi assays, the ovaries and carcasses were grouped into threes, while for all other assays individual ovaries and carcasses were used. Genomic DNA (gDNA) was extracted using the crude STE boiling method (O'Neill et al. 1992). Tissues were homogenized using a mortar and pestle in 50 µL STE, then treated with two µL of 10 mg/mL proteinase K (Roche) and incubated at 55° C for one hour, followed by 15 minutes at 95° C to inactivate the enzyme. The gDNA was used to perform real-time qPCR using standardized procedures. Quantification was performed on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, California, United States) at the Michigan State University Genomics Core, using a Quantitect SYBR Green PCR Kit (Qiagen). Five independent replicates were performed for RNAi assays, 10 for bacterial challenges, and six for all other experiments, and reactions were performed in duplicate. Primer sequences used are found in Table 2.1. Standard curves were created for *Wolbachia* type B

specific *wsp* using a plasmid from Tortosa et al. (2008) and *RPS6* using a plasmid created previously in our lab.

RNA interference assays. RNA interference (RNAi) was used to perform gene-silencing assays according to standard procedures (Dong et al. 2006). Mosquito total RNA was collected from whole mosquitoes using a Qiagen RNEasy kit (Qiagen, Germantown, MD USA) according to the manufacturer's protocol. Clone DNA (cDNA) was created from this RNA using a QuantiTect Reverse Transcriptase Kit (Qiagen), and a section of the cDNA complementary to the gene of interest was amplified with PCR. The PCR product was extracted with a QIAquick Spin Kit (Qiagen) and used to create double stranded RNA (dsRNA) with a T7 MEGAScript Kit (Ambion, Foster City, CA USA) followed by purification by phenol/chloroform extraction. For single knockdown, 69 nL of the dsRNA at 4 µg/µL was injected into the thorax of two-day old adult female mosquitoes anesthetized with CO_2 using a Nanoject II (Drummond Scientific, Broomall, PA USA); for double gene knockdown 138 nL were injected.

Five types of dsRNA were prepared against different genes: green fluorescent protein (*GFP*), *Cactus*, *Caspar*, *Rel1*, and *Rel2*. The primers for these genes are found in table 2.1. dsRNA of *GFP*, a gene not present in mosquitoes, was used as a negative control. *Cactus* and *Caspar* knockdown induced the Toll and Imd pathways, respectively, while *Rel1* and *Rel2* knockdown repressed the Toll and Imd pathways, respectively. Double knockdown assays were also performed, using a combination of *Cactus* and

Gene	Purpose	Primer Sequence (forward/reverse)
Caspar	dsRNA	TAATACGACTCACTATAGGGGCTCAAGTCGCTCAACATAGG / TAATACGACTCACTATAGGGATCGTGTAGACGTCGGTTTTG
Cactus	dsRNA	TAATACGACTCACTATAGGGGGAAGCAGATCGAGCCAAGCAG / TAATACGACTCACTATAGGGGCATTGAGCCGCCTGGTGTC
Rel1	dsRNA	TAATACGACTCACTATAGGGTGGTGGTGGTGTCCTGCGTAAC / TAATACGACTCACTATAGGGCTGCCTGGCGTGACCGTATCC
Rel2	dsRNA	TAATACGACTCACTATAGGGGCTCAGTGCTACCGTGGGAAAC / TAATACGACTCACTATAGGGCGGGTTCGCTCTGGCATTTGTC
GFP	dsRNA	TAATACGACTCACTATAGGGGGAGAAGAACTTTTCACTGG / TAATACGACTCACTATAGGGAGTTGAACGGATCCATCTTC
wsp	PCR	TGGTCCAATAAGTGATGAAGAAAC / AAAAATTAAACGCTACTCCA
*w*AlbB *wsp*	qPCR	AAGGAACCGAAGTTCATG / AGTTGTGAGTAAAGTCCC
RPS6	qPCR	CGTCGTCAGGAACGTATCCG / TCTTGGCAGCCTTAGCAGC
Rel1	qPCR	TGGTGGTGGTGTCCTGCGTAAC / CTGCCTGGCGTGACCGTATCC
Rel2	qPCR	GCTCAGTCGTACCGTGGGAAAC / CGGGTTCGCTCTGGCATTTGTC

Table 2.1: Primers used to create double stranded RNA for RNAi assays and for real-time qPCR.

Caspar or *Rel1* and *Rel2* to induce or repress both pathways at the same time. Double knockdown of *Rel1* and *Rel2* had to be performed separately from the individual knockdowns of those genes due to high mosquito mortality. Six days after dsRNA injection, mosquitoes were sacrificed and dissected for real-time qPCR as described above.

Rearing of aseptic mosquitoes: Ae. aegypti mosquitoes of the WB1 strain were hatched and reared at low densities as described above. Upon eclosion, the mosquitoes were housed in a sterilized cage and fed autoclaved 10% sucrose solution containing 15 mg/mL gentamicin, 10 units of penicillin and 10 µg streptomycin. After six days, mosquitoes were sacrificed for real-time qPCR as described above. To confirm the removal of microbes, mosquitoes were surface sterilized by washing in 70% ethanol followed by two rinses in double distilled water. Midguts were dissected from mosquitoes in autoclaved PBS. Whole mosquitoes and midguts were homogenized in 100 µL of autoclaved PBS and plated on LB-agar plates. After 24 h of incubation, plates were visually checked for growth.

Bacterial and fungal challenges: Ae. aegypti mosquitoes of the WB1 strain were hatched and reared as described above. One day after eclosion, female mosquitoes were injected with gram positive (*Micrococcus luteus*) or gram negative (*Enterobacter cloacae*) bacteria in lysogeny broth (LB), fungus (*Beauveria bassiana*) or sterile LB using an acupuncture needle dipped in the

solution to penetrate the mosquito abdomen. Mosquitoes were sacrificed prior to infection, and at five and ten days after infection and used for real-time qPCR as described above.

Dengue virus-2 infections: Dengue virus type-2 of the New Guinea C strain was propagated in the *Ae. albopictus* C6/36 cell line and used to provide the mosquitoes with an infectious blood meal according to standardized procedures. C6/36 cells were grown to 80% confluency at 32° C and 5% CO_2 in 25 cm^2 flasks using minimal essential medium (MEM) with 10% heat inactivated FBS, 1% L-glutamine and 1% non-essential amino acids. 0.5 mL of virus stock was used to infect these flasks to a multiplicity of infection (MOI) of 3.5 virus particles per cell, and the cells were incubated for seven days. Infected cells were then harvested using a cell scraper, spun down in a centrifuge and lysed by repeated freeze/thaw cycles using dry CO_2 mixed with 100% ethanol to quickly freeze and a 37° C water bath to quickly thaw the cells. The virus suspension was mixed with an equal volume of defibrinated sheep blood and used to feed one-week old mosquitoes using a water bath to maintain the blood at 37° C as described previously (Das et al. 2007). To provide comparison, mosquitoes were also provided a blood meal using the same blood mixed with an equal proportion of only MEM to serve as an uninfectious blood meal. Mosquitoes were sacrificed immediately before the blood meal and five, ten, and 14 days post-blood meal. *Wolbachia* levels were quantified with real-time qPCR as described above.

Creation of Wolbachia-*infected transgenic lines:* Transgenic mosquitoes overexpressing *Rel1* or *Rel2* under the control of the vitellogenin promoter were obtained from the Raikhel laboratory at The University of California Riverside. These mosquitoes were hatched and larvae reared according to the protocol outlined above. Upon eclosion, virgin males were mated with virgin females of the WB1 strain, creating *Wolbachia* infected offspring bearing the transgene. Virgin females of the F1 group were backcrossed with virgin transgenic males, yielding offspring with approximately 75% genetic similarity to the original transgenic mosquitoes, but bearing a *Wolbachia* infection. At each stage, larvae were screened for *GFP* expression in their eyes so that only mosquitoes expressing the transgene were used for breeding and experimentation.

After *Wolbachia*-infected transgenic mosquito lines were created, their *Wolbachia* infection densities were measured. The percent infected was monitored for six generations. To measure the *Wolbachia* densities, seven day old mosquitoes were provided blood meals from ketamine-anesthetized mice to induce expression of the transgenes. Six individual female mosquitoes were sacrificed and their ovaries and carcasses collected just prior to each blood meal, and again seven days after the second blood meal; samples were of mosquitoes aged seven, 14 and 21 days. Genomic DNA was extracted from each sample and used for real-time qPCR according to the procedures outlined above. Each time samples were collected, whole body total RNA was collected using an RNEasy Blood and Tissue Kit according to manufacturer's instructions (Qiagen). This RNA was used to make cDNA with a Qiagen Reverse Transcriptase Kit

(Qiagen) and used for real-time qPCR to check the expression levels of the genes of interest using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen 2001).

Results

RNAi assays: In order to study the role of mosquito immunity on regulation of *Wolbachia* density, we used RNAi to up- or down-regulate the Toll and Imd pathways and measured the impact on *Wolbachia* infection levels. We observed a significant increase in *Wolbachia* density in the ovaries of mosquitoes injected with a combination of *Cactus* and *Caspar* dsRNA relative to the *GFP* injected control ($p<0.05$, Mann-Whitney). No other significant effects were observed in either the ovaries or remaining carcasses of mosquitoes with upregulated immune systems by injection of *Cactus* or *Caspar* dsRNA, and there was no effect on *Wolbachia* levels in mosquito carcasses after injection with both dsRNAs (Figure 2.1). Of mosquitoes with downregulated immune systems by injection of *Rel1* or *Rel2* dsRNA, only the ovaries of those injected with *Rel2*, which suppressed the Imd pathway, differed significantly from the *GFP* control with significantly lower *Wolbachia* densities ($p<0.05$ Mann-Whitney) (Figure 2.2). All other single knockdown treatments in both ovaries and carcasses showed no significant difference (Figure 2.2). Mosquitoes with both immune pathways reduced through injection of both *Rel1* and *Rel2* dsRNA experienced approximately 80% mortality. For the surviving mosquitoes, we observed no difference in *Wolbachia* density relative to the *GFP* injected control in either ovaries or carcasses (Figure 2.3).

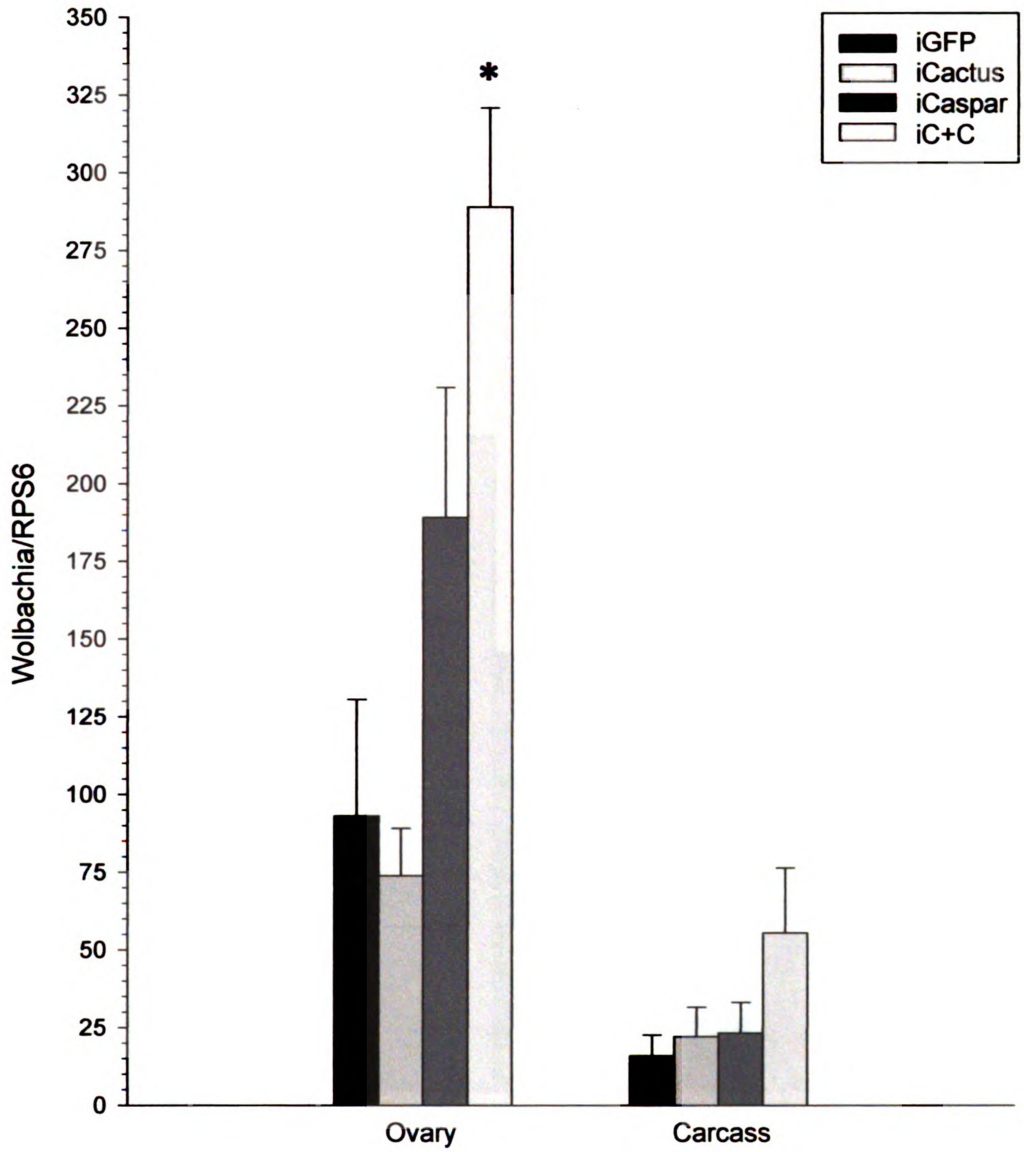

Figure 2.1: *Wolbachia* densities in mosquitoes after induction of immune pathways through RNAi. Induction of both the Toll and Imd pathways by RNAi causes decreased *Wolbachia* in mosquito ovaries but has no effect in the remaining carcass. * indicates a significant difference from the GFP injected control (Mann-Whitney, α=0.05). Error bars represent standard error of the mean.

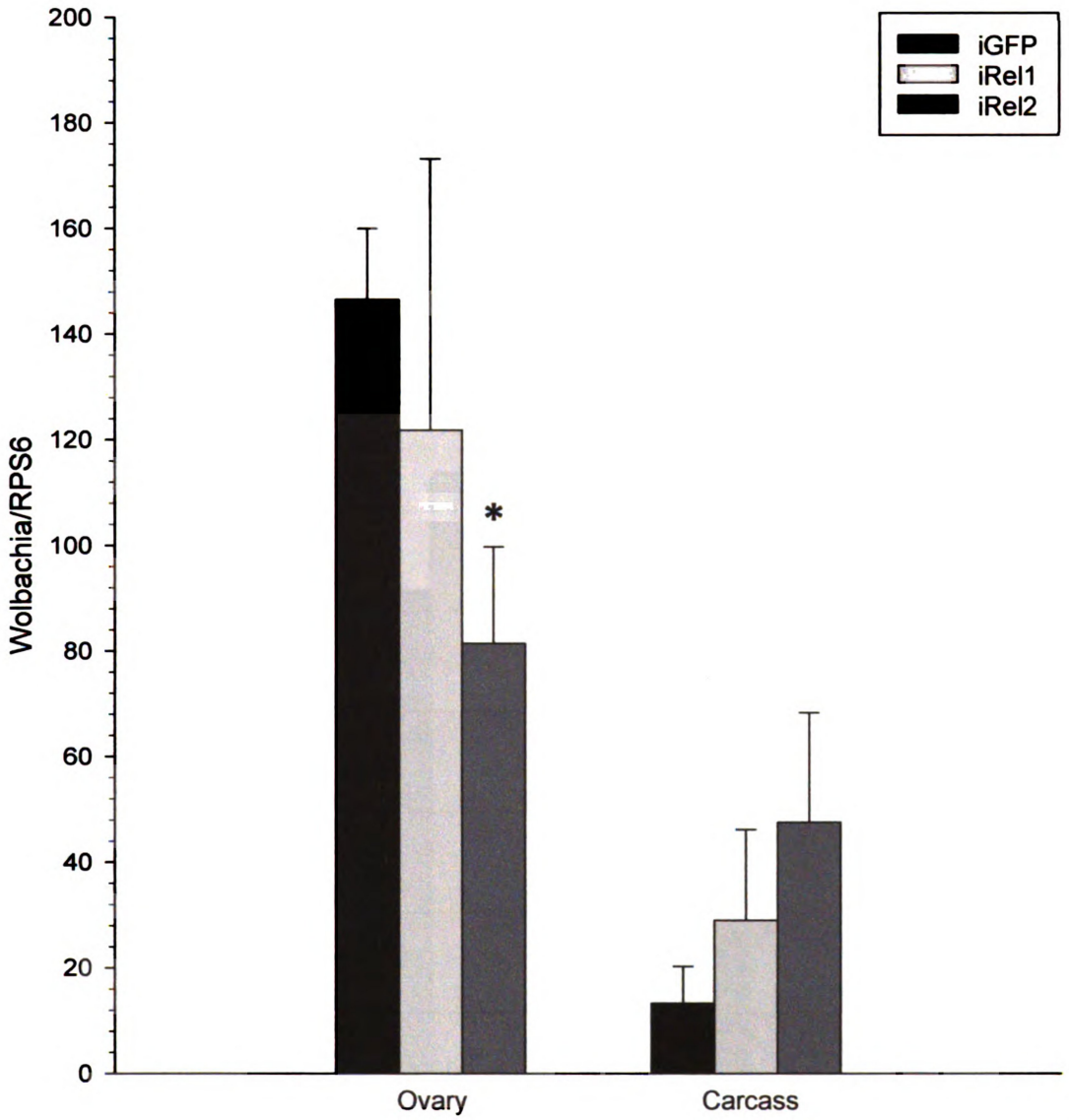

Figure 2.2: *Wolbachia* densities in mosquitoes after reduction of single immune pathways through RNAi. Reduction of the Imd pathway through RNAi decreases the *Wolbachia* density of mosquito ovaries, but has no effect on densities in the mosquito carcass. * indicates a significant difference from the GFP injected control (Mann-Whitney, α=0.05). Error bars represent standard error of the mean.

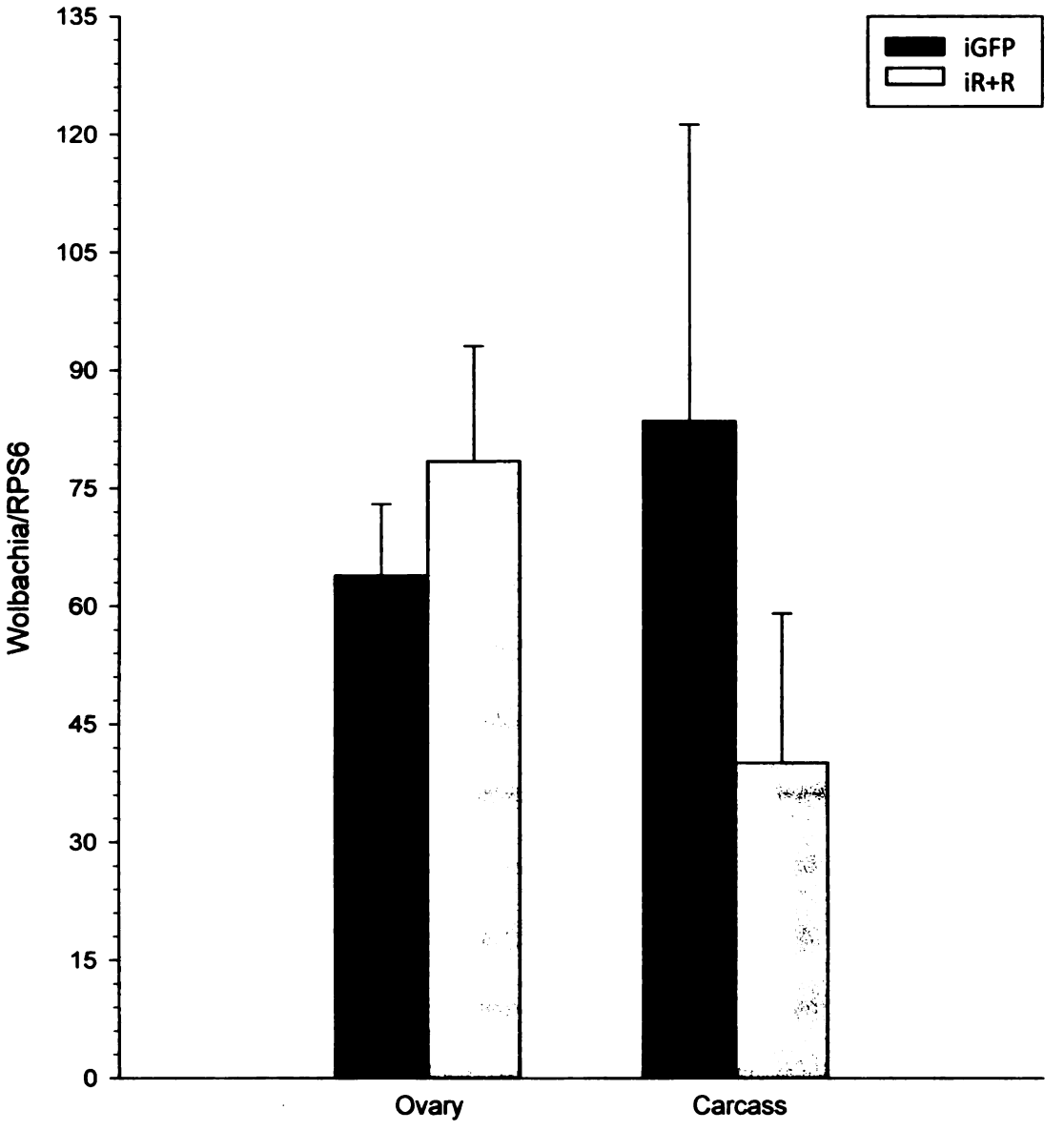

Figure 2.3: *Wolbachia* densities in mosquitoes after reduction of both immune pathways through RNAi. Reducing both the Toll and Imd pathway simultaneously through RNAi has no significant effect on *Wolbachia* density in either ovaries or carcasses of surviving mosquitoes (~20% of total injected mosquitoes). Error bars represent standard error of the mean.

Rearing of aseptic mosquitoes: To investigate how a mosquito's endogenous flora affects *Wolbachia* levels, we fed mosquitoes with antibiotic treated sucrose and measured how *Wolbachia* densities changed. Plating of bacteria from mosquito midguts and whole bodies confirmed that the antibiotics were effective at removing most of the natural mosquito microbiota (data not shown). However, no difference in the *Wolbachia* density of WB1 mosquitoes was observed in either ovaries or carcasses between mosquitoes treated with antibiotics and those not treated with antibiotics (Figure 2.4).

Bacterial and fungal challenges: In order to explore how bacteria and fungi can affect *Wolbachia* densities, we infected mosquitoes with gram-negative or gram-positive bacteria or fungi and measured changes in *Wolbachia* levels. Before infection and five days after, there were no significant differences in *Wolbachia* densities between any challenged group and the LB injected control group in either mosquito ovaries or carcasses (Figure 2.5). Ten days after challenge, however, there were significantly fewer *Wolbachia* in the ovaries of mosquitoes infected with the gram-negative bacteria *E. cloacae* than in the ovaries of the control mosquitoes ($p<0.05$, Mann-Whitney) (Figure 2.5a). No other significant differences were observed on day ten post-infection.

Dengue virus-2 infections: In order to observe the effects dengue virus infection has on *Wolbachia* infection, we infected mosquitoes with dengue virus and measured the changes in *Wolbachia* densities. There was no significant

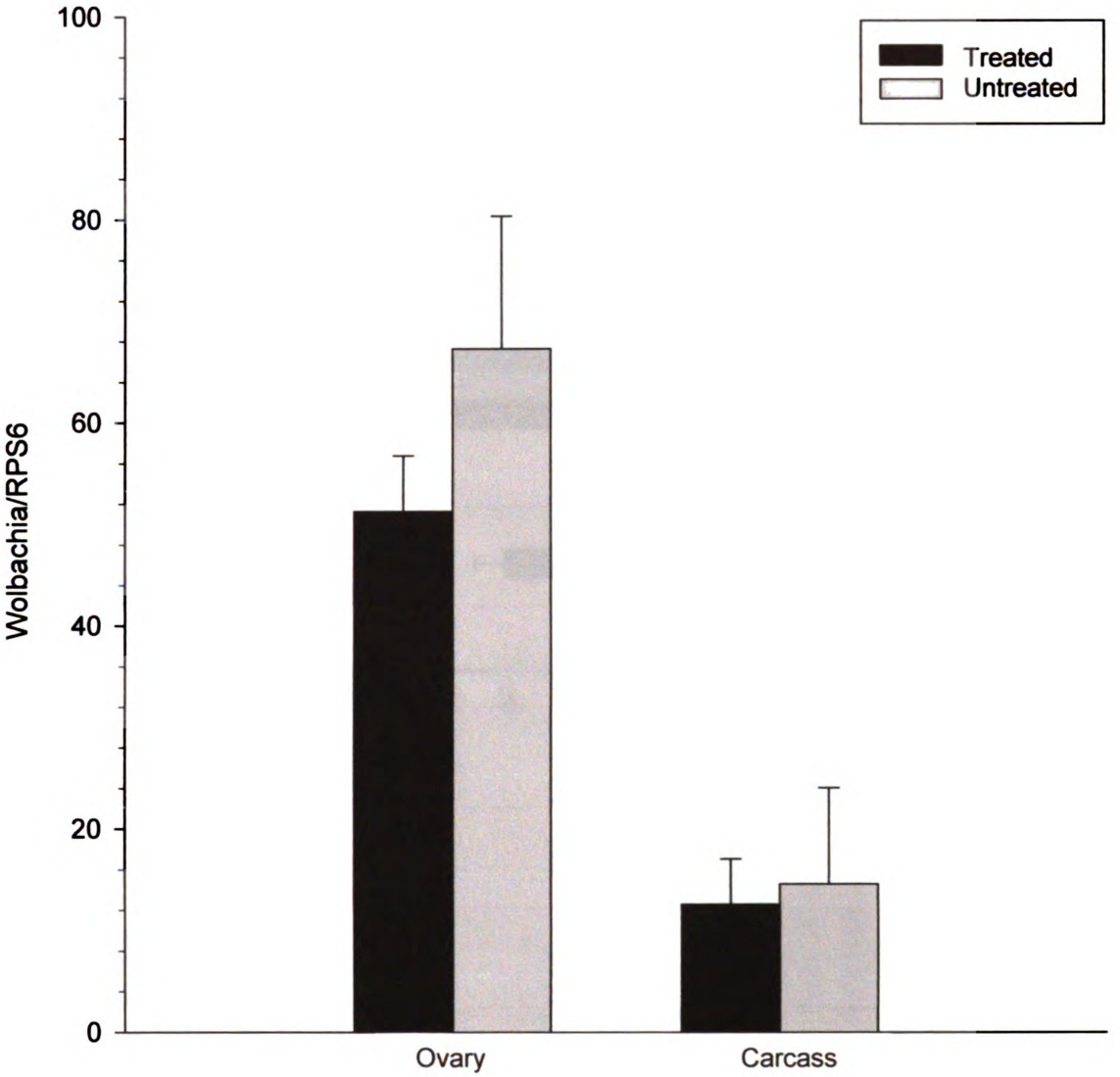

Figure 2.4: *Wolbachia* densities in mosquitoes 6 days after treatment with antibiotics through adult feeding. Removal of the mosquito endogenous flora through antibiotic treatment has no effect on the *Wolbachia* densities in either the mosquito ovaries or carcasses. Error bars represent standard error of the mean.

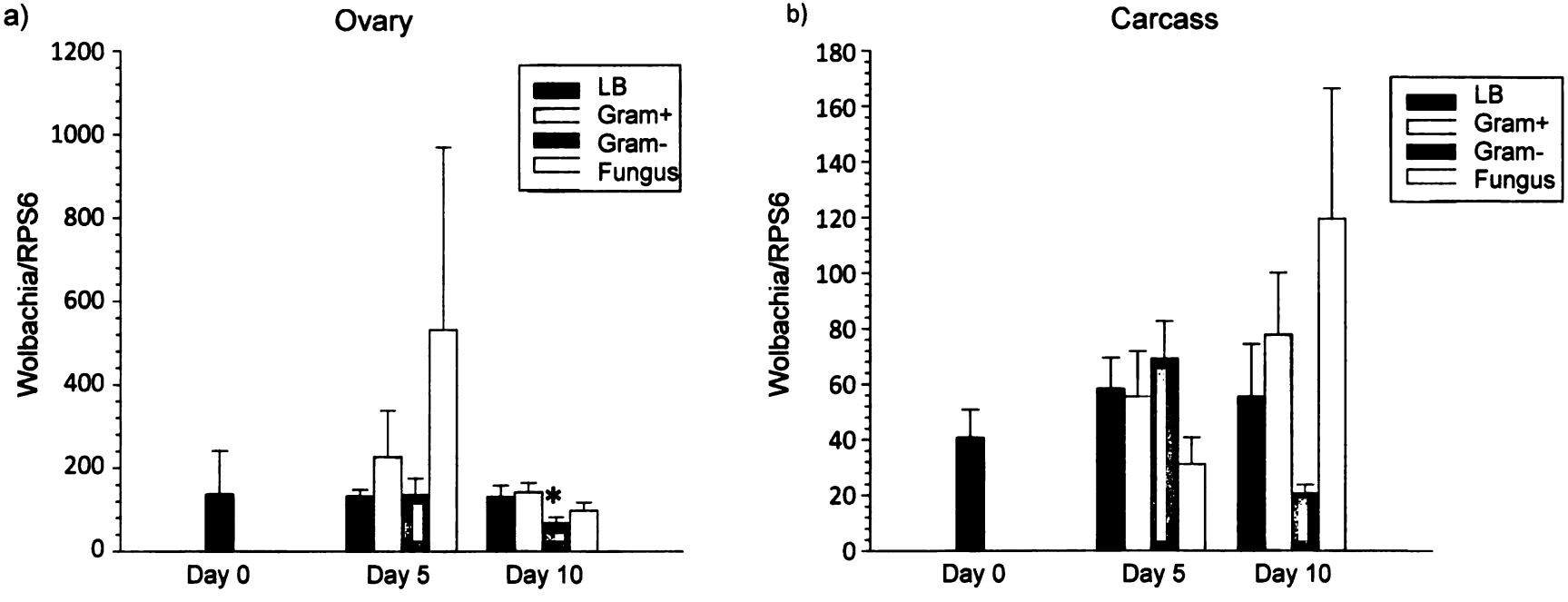

Figure 2.5: *Wolbachia* densities in mosquito ovaries (a) and carcasses (b) before and 5 and 10 days after infection with gram positive bacteria, gram negative bacteria or fungi. Mosquitoes infected with gram negative bacteria had significantly lower *Wolbachia* densities in their ovaries than LB-injected control mosquitoes 10 days post infection. No other infections had a significant effect on the *Wolbachia* densities at any time, and there was no difference in carcass *Wolbachia* densities throughout the study. * indicates a significant difference from LB injected control (Mann-Whitney, α=0.05). Error bars represent standard error of the mean.

difference in *Wolbachia* density observed between dengue virus-infected and uninfected mosquitoes in either ovaries or carcasses 14 days after their blood meal (Figure 2.6).

Creation of Wolbachia *infected transgenic lines:* To further examine how mosquito immunity can affect *Wolbachia* densities, we created *Wolbachia* infected transgenic mosquito lines overexpressing *Rel1* or *Rel2* and quantified the *Wolbachia* densities after activating the gene expression. Our crossing experiments were able to create mosquitoes bearing the transgene and a stable *Wolbachia* infection as confirmed by standard PCR using general *wsp* primers 81F and 691R (Zhou et al. 1998). These lines were named *Rel1*B1, *Rel2*B1, and UgalB1. This infection continued throughout the study in all three lines, and was confirmed each generation up to six generations after creation (Figure 2.7). When *Wolbachia* levels were tested prior to the first blood meal, the *Wolbachia* density was significantly higher in the ovaries of mosquitoes overexpressing both the *Rel1* and the *Rel2* gene than in control UgalB1 mosquitoes ($p<0.05$, Mann-Whitney) (Figure 2.8a). One week after both the first and second blood meals there was no significant difference in *Wolbachia* densities in mosquito ovaries between the control and experimental mosquitoes. At no point during the study was there a significant difference in the *Wolbachia* densities of transgenic and control mosquito carcasses (Figure 2.8b).

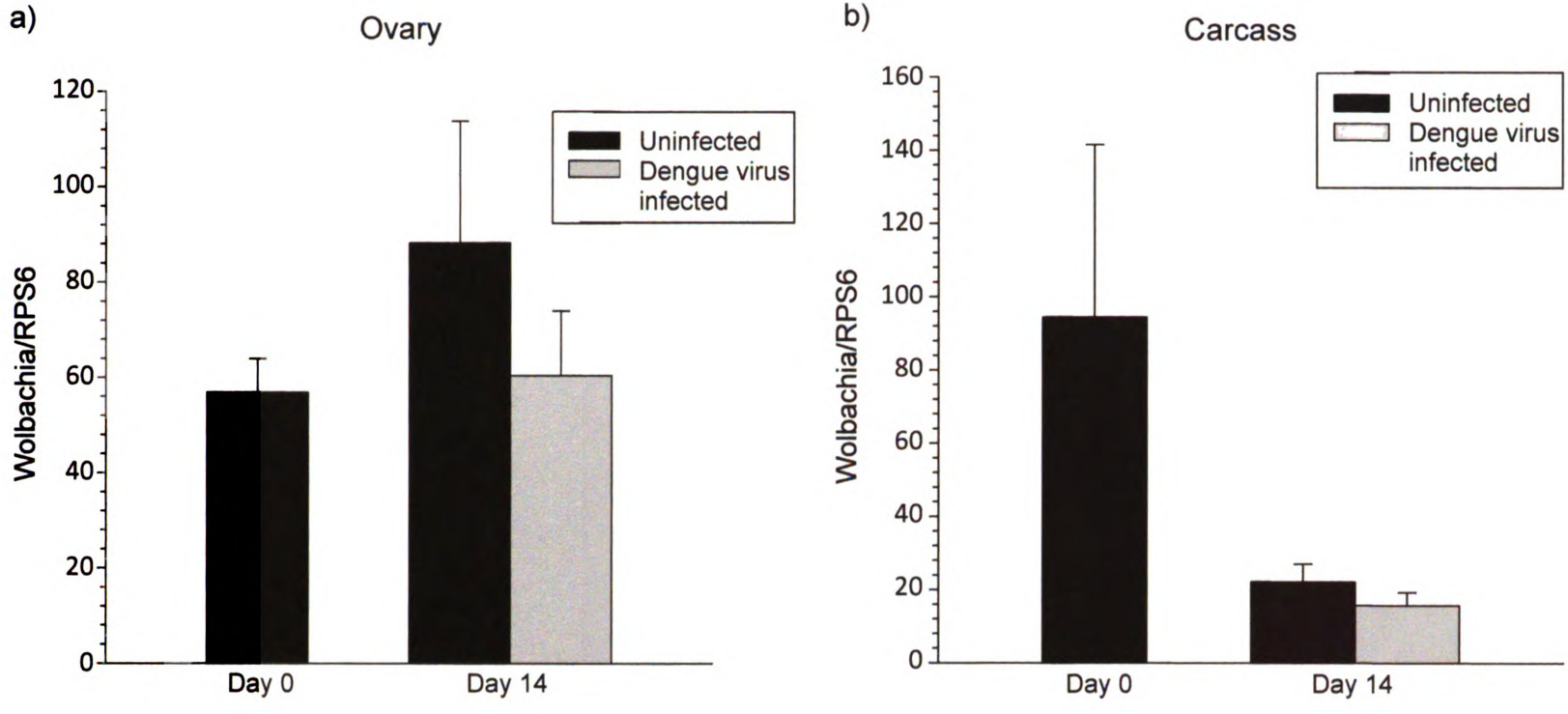

Figure 2.6: *Wolbachia* densities in mosquito ovaries (a) and carcasses (b) before and after a dengue uninfected or infected blood meal. Dengue virus infection had no significant effect on the *Wolbachia* densities of mosquito ovaries or carcasses 14 days post-blood meal. Error bars represent standard error of the mean.

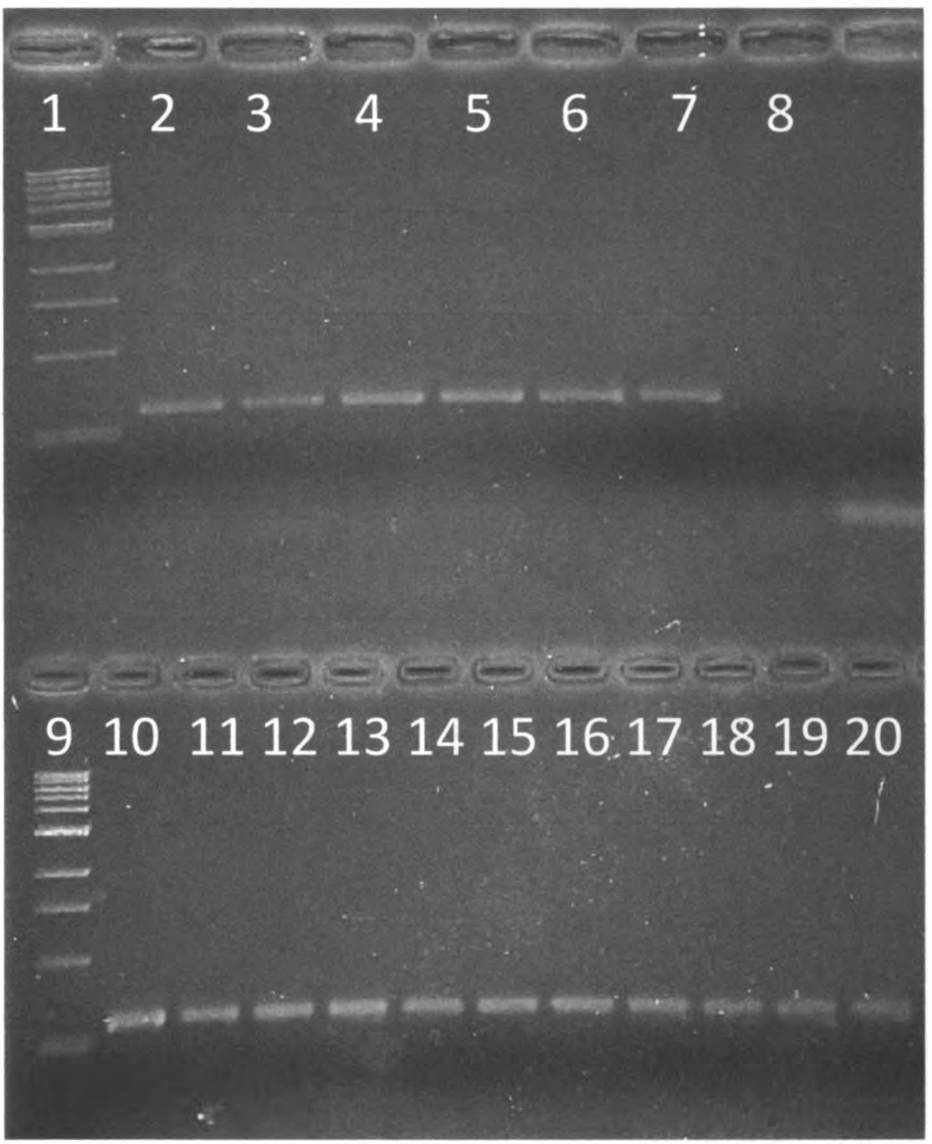

Figure 2.7: PCR product confirming *Wolbachia* infection in transgenic mosquito lines six generations after introgression. Lane 1: 1 kb ladder, Lanes 2-6: UgalB mosquitoes, Lane 7: positive control, Lane 8: negative control, Lane 9: 1 kb ladder, Lanes 10-14: Rel1B mosquitoes, Lanes 15-19: Rel2B mosquitoes, Lane 20: positive control.

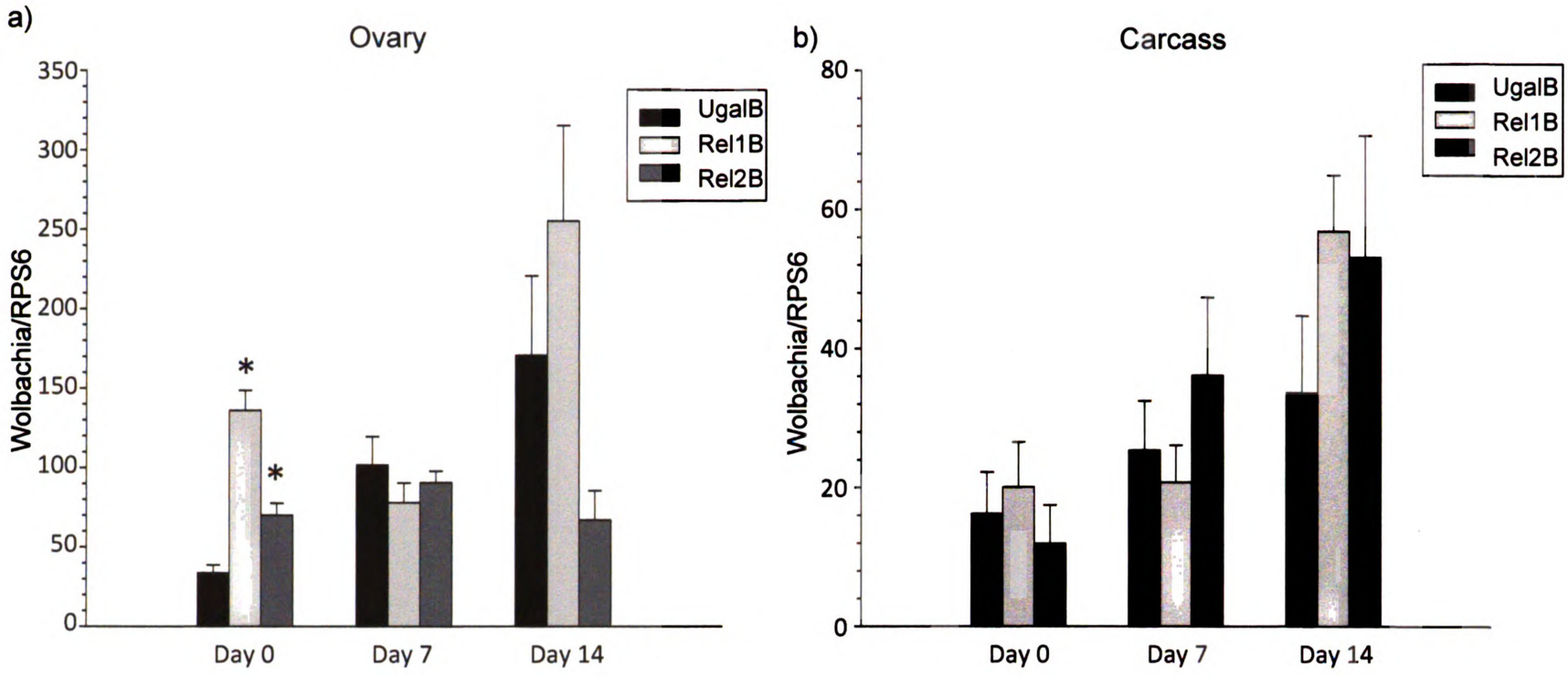

Figure 2.8: *Wolbachia* densities in transgenic mosquito ovaries (a) and carcasses (b) before a blood meal and 7 and 14 days after a blood meal. Transgenic mosquitoes overexpressing either *Rel1* or *Rel2* had significantly higher ovary *Wolbachia* densities than wild-type mosquitoes before a blood meal, but 7 and 14 days after blood meals there was no difference. There was also no difference in *Wolbachia* density in mosquito carcasses at any time. * indicates a significant difference from wild type mosquitoes (Mann-Whitney, α=0.05). Error bars represent standard error of the mean.

Discussion

The fact that *Ae. aegypti* does not naturally carry *Wolbachia* but is able to be artificially infected provides a unique opportunity to develop *Wolbachia*-based intervention strategies for dengue control (Xi et al. 2005, McMeniman et al. 2009). Such control strategies require an understanding of the stability of *Wolbachia* infection in this, the primary vector of dengue virus, especially considering the evidence supporting a previous loss of an ancient *Wolbachia* infection in this mosquito species. Because *Wolbachia*'s effectiveness as a gene driver and disease control mechanism is reliant on its ability to induce CI and that ability may be linked to the density of *Wolbachia* in the insect, it is important to determine what factors are influencing *Wolbachia* levels (Duron et al. 2009, Unckless et al. 2009). Our results indicate that the mosquito immune system and the microbiota inhabiting the mosquitoes are able to influence *Wolbachia* density levels to a significant degree.

The mosquito immune system was able to affect *Wolbachia* levels significantly. Specifically, simultaneous activation of both the Toll and Imd pathways and suppression of the Imd pathway alone were able to increase and decrease *Wolbachia* densities in the mosquito ovaries, respectively. Because the mosquito immune system is able to modulate infection by bacteria, viruses, and other parasites, it is not surprising that there is an interaction between the mosquito immune system and *Wolbachia* densities (Dimopoulos et al. 1997, Xi et al. 2008a). However, that the induction of the immune pathways leads to an increase in ovarian bacterial densities was unexpected. This lends support to the

hypothesis that *Wolbachia* interacts with other microbes inhabiting the same host are mainly through competition. Inducing the mosquito immune system with RNAi could reduce the levels of numerous other microbes inhabiting the mosquitoes, thereby removing competitive pressure from the *Wolbachia* and allowing the densities to increase. Similarly, the fact that reduction in the Imd pathway leads to a reduction in *Wolbachia* can be explained by reduced immune response leading to an increase in other microbes, increased competition, and thus less *Wolbachia*. This study has not proven this, however, as we did not measure the densities of other microbes.

While we saw a significant decrease in *Wolbachia* densities after injection of dsRNA targeting *Rel2* and reducing the Imd pathway, we observed no such effect after injection of dsRNA targeting both *Rel1* and *Rel2*. It would be expected that if repressing one pathway could lead to a decrease in *Wolbachia* and repressing the other does not lead to an increase in *Wolbachia*, repressing both should also lead to a decrease in *Wolbachia*. This is likely due to the increased death of mosquitoes injected with both type of dsRNA. With both the Imd and Toll pathway downregulated, the mosquitoes had greatly reduced immune capacities to protect them, and approximately 80% of them died. Those that did survive were likely those with lower levels of repression of their immune systems and therefore lack the impact on growth of microbial flora. We would expect those individuals to have the least change in *Wolbachia* densities if the competition hypothesis is correct.

We observed that *Wolbachia* levels were higher in transgenic mosquitoes before the blood meal than in wild-type mosquitoes, but the effect disappeared after the blood meals. One possible explanation for this is that the mosquito flora has been permanently changed by adapting to many generations of exposure to the transgenes. Because the mosquitoes need blood meals to reproduce but the blood meals increase immune response, each time the mosquitoes are bred their flora is exposed to the increased immune response. Thus, over time, poorly adapted microbes would be removed from those mosquitoes permanently, while the remaining flora would be adapted to the immune effector genes. Future overexpression of those immune genes would have diminished effect on the mosquito flora, which results in no change of the *Wolbachia* levels.

Alternatively, this pattern could indicate that there is a "leaky system" in which the transgenes are slightly overexpressed before induction by the blood meal, causing a reduction in microbial populations and an increase in *Wolbachia* as above. After the blood meals, however, the immune response was so much greater in transgenic mosquitoes than in wild-type mosquitoes that the immune system could be acting directly against the *Wolbachia* rather than indirectly through competitive effects. This would explain the pattern of *Rel2*B mosquitoes having the lowest *Wolbachia* levels 14 days after the first blood meal. While not significantly different from wild-type mosquitoes, these mosquitoes did appear to have a trend of reduced *Wolbachia* densities, perhaps because the Imd pathway, which is turned on by *Rel2*, acts against gram-negative bacteria, including *Wolbachia*. That these effects were not observed during the RNAi experiments

could be because RNAi assays have less of an effect on the genes they target than the creation of transgenic mosquitoes does. RNAi effects may be too weak to act directly against the *Wolbachia*, being limited to indirect effects through competition.

It is also possible, but unconfirmed, that *Wolbachia* may be able to evade the host immune system to a certain point. This would give them a competitive advantage over other microbes inhabiting the same host, but could fail when the immune genes are highly upregulated, as in our transgenic mosquitoes. *Wolbachia* infecting *Nasonia vitripennis* wasps may modulate their host response with ANK-PRANC genes that are known to reduce NF-κB in mammals (The Nasonia Genome Working Group 2010). If all *Wolbachia* are able to modulate their host immunity in this way, it could explain why they apparently escape the effects of increased immunity while other gram-negative bacteria do not.

Our results from the pathogen challenges parallel results found in other studies investigating the effects of co-infection of *Wolbachia* and another pathogen. For instance, in *Ae. albopictus Wolbachia* densities were slightly decreased when the mosquitoes become infected with Chikungunya virus (Tortosa et al. 2008). While we saw no difference in dengue-infected mosquitoes, we did notice that mosquitoes infected with gram-negative bacteria had decreased *Wolbachia* levels. This could indicate competition in which *Wolbachia* and the pathogen both utilize the same limited host resource, causing *Wolbachia* densities to decrease. It has also been suggested that mosquitoes may decrease their *Wolbachia* infection levels in response to stress, although the

mechanism is unknown (Berticat et al. 2002). This again would be consistent with our results, as mosquitoes put under the stress of infection by other bacteria would moderate their *Wolbachia* infection. However, the lack of a suggested mechanism leaves this hypothesis untested.

The competition hypothesis is not supported by our observation of no effect on *Wolbachia* infection density by the removal of microbial flora. One possible explanation for this is that the antibiotics we used were specifically chosen to limit their effects on *Wolbachia* while removing midgut flora. Other intracellular bacteria, living near *Wolbachia* and possibly in greatest competition with them, may also have avoided the antibiotics we used. Also, the method of feeding antibiotic treated sucrose to adult mosquitoes is not able to remove all of the bacteria in the mosquitoes, as evidence by the fact that there were still some bacteria growing on the plates we used to check their microbial flora. Our plates showed a greater than 50% reduction in bacterial growth in mosquitoes fed with antibiotics, but the plates still had some bacteria growing. Therefore, the antibiotics may not have been able to remove the endogenous bacteria to the extent that there is an effect on the *Wolbachia* levels.

These data may also provide support for the competition theory of virus attenuation in both mosquitoes and *Drosophila*. Multiple studies have confirmed that *Wolbachia* infection can remove the negative effects of infection by many human and insect pathogens without offering a mechanism for how it occurs (McGraw et al. 2002, Hedges et al. 2008, Moreira et al. 2009b, Bian et al. 2010). If competition is the mechanism by which *Wolbachia* levels are controlled in

insect hosts, it may also be the way in which viruses are being limited when co-infecting a host with *Wolbachia*. Again, this study does not address this question, but it provides evidence that mosquitoes are able to control their microbiota through indirect interactions with their immune system rather than solely by direct immune attacks.

The observed effects in *Wolbachia* density occurred in the ovaries of the mosquitoes, while the *Wolbachia* densities in the remaining carcasses went unchanged. This is significant, as the reproductive organs are the locations in which any reproductive modifications occur, and the strength of such modifications can be directly related to *Wolbachia* densities. If the reproductive tissues were able to maintain high levels of *Wolbachia* while the densities in the rest of the body dropped, we would expect little difference in the expression of any reproductive modifications. However, we observed the opposite pattern, indicating that if *Wolbachia* density is related to the strength of CI in *Ae. aegypti*, these changes in density will likely have an effect on the phenotype, and thus on the usefulness of *Wolbachia* as a control mechanism.

However, it is not yet known how, if at all, *Wolbachia* densities affect CI in *Ae. aegypti*. *Culex* spp. mosquitoes exhibit CI independent of *Wolbachia* density, while *Drosophila* spp. and *Nasonia* wasps tend to have decreased reproductive effects with lower *Wolbachia* densities (Breeuwer and Werren 1993, Sinkins et al. 1995, Perrot-Minnot et al. 1996, Duron et al. 2009, Unckless et al. 2009). Therefore, it is possible that decreased *Wolbachia* densities in *Ae. aegypti* could cause reduced CI, but it must be confirmed. Decreased *Wolbachia* densities in

the reproductive tissues could also affect maternal transmission rates, even if the strength of reproductive modification remains high. If some eggs were laid without any *Wolbachia*, those offspring would start a *Wolbachia* uninfected population among the infected mosquitoes, which could lead to a loss of *Wolbachia* in the population. If *Wolbachia* are bearing a gene making their host refractory to a disease through paratransgenesis, this loss would cause the vector control program to fail. Even if *Wolbachia* were used only to drive transgenes in other co-infecting microbes into a population, this loss would break the linkage between the transgene and the gene driver, again causing the control strategy to fail.

It is still unknown what the real impacts of these effects are on natural populations. It is possible that over multiple generations the *Wolbachia* infection level can rebound from the depletion caused by an increase in a competing bacterium. This would indicate that *Wolbachia* remain a strong choice for a gene drive mechanism in mosquitoes. Therefore, it will be important in the future to determine whether these effects are removed in successive generations or not. Also, the *Wolbachia* infection in *Ae. aegypti* is a relatively recent association, as the WB1 strain has only been maintained for approximately six years (Xi et al. 2005). Over time, the effects of *Wolbachia* infection on their host can change, which may indicate co-adaptation of the host to the symbiont (Carrington et al. 2010). If this is the case, it is possible that, over time, the effects of the mosquito immune system on *Wolbachia* infection may be reduced or removed, making this less of an issue. Only observation over a long time period will prove or disprove

this theory. Also, these results are only for one species of vector mosquito. Many other important vector species have also been suggested as targets of *Wolbachia*-based control programs, and their interactions with *Wolbachia* may be different. Similarly, *Wolbachia* infection dynamics may be different in the field than the laboratory, as *Wolbachia* densities also depend on numerous other factors, such as larval densities and temperatures, which were kept constant in the laboratory.

Overall, we have observed that the mosquito immune system is able to affect *Wolbachia* densities in *Ae. aegypti* ovaries. Consistent with that, infection with Gram negative bacteria can reduce *Wolbachia* density in mosquito ovaries. If *Wolbachia* density proves to be correlated with CI strength in *Ae. aegypti*, these results will have important implications for the feasibility of using *Wolbachia* to control dengue virus spread. The fact that *Wolbachia* can potentially be used both as a gene driver and as an effector itself has made it one of the more promising new mosquito control methods. Before it can be put into effect, the interactions between the mosquito, *Wolbachia,* and any pathogen must be better understood to ensure that the program will not fail. If the mosquito immune system or microbiota can remove *Wolbachia*, any control method based on this system will likely fail. However, if *Wolbachia* were able to outcompete other microbes, their usefulness for control will only increase.

CHAPTER 3

Effects of co-infection by *Wolbachia* and dengue virus on mosquito life history

Introduction

Multiple authors have suggested that there is no cost to insects of transmitting arboviruses, arguing that any negative effects on the vector would reduce the efficiency of transmission (Chamberlain and Sudia 1961, Hardy et al. 1983). This hypothesis has been upheld by studies that have found no effect of infection by dengue virus or La Crosse virus (Patrican and DeFoliart 1985, Putnam and Scott 1995). However, recent studies have shown that mosquito infection by West Nile virus or dengue virus can impact mosquito fecundity, lifespan, and behavior (Platt et al. 1997, Styer et al. 2007, Bian et al. 2010). Infection by endosymbionts such as *Wolbachia* can also have significant impacts on mosquito lifespan and behavior, though the effects vary by host species (Dobson et al. 2004, Moreira et al. 2009a, Suh et al. 2009, Turley et al. 2009). Few studies have investigated the effects of infection by both a pathogen and a symbiont on insects; however, it has been shown that in *Drosophila melanogaster* and *Aedes aegypti*, virus infection levels and the negative effects of infection are reduced by the presence of *Wolbachia* (Hedges et al. 2008, Moreira et al. 2009b, Bian et al. 2010).

Dengue virus, the causative agent of dengue fever and the deadly dengue hemorrhagic fever, is likely the most important arbovirus in the world today. Each year 50-100 million cases occur, and it is estimated that 2.5 billion people are currently at risk for contracting the virus (WHO 2009). The virus is

transmitted by the mosquitoes *Ae. aegypti* and *Ae. albopictus,* and the lack of an effective vaccine or specific treatment leaves vector control strategies as the primary intervention tool for these serious diseases. Traditional vector control strategies, including bed nets, pesticide treatments, and habitat reduction have been in place for years, but have been unable to effectively stop disease transmission (Kyle and Harris 2008). Instead, recent efforts have focused on the creation of mosquitoes refractory to disease transmission, with some success (Olson et al. 1996, Franz et al. 2006). However, once these mosquitoes are created they will still need to be driven into the population. To do this, numerous gene drive mechanisms have been suggested, including the endosymbiotic bacterium *Wolbachia*.

Wolbachia are maternally inherited alphaproteobacteria, found in up to 66% of insect hosts, which cause a variety of reproductive modifications in their hosts including male-killing, induction of parthenogenesis, and cytoplasmic incompatibility (CI) (Hilgenboecker et al. 2008, Werren et al. 2008). CI is the phenotype found in mosquitoes, and is a condition in which the embryos created by matings between *Wolbachia*-infected males and uninfected females fail to develop, while infected females are able to mate successfully with any males. This gives infected females a reproductive advantage over their uninfected counterparts and can facilitate the spread of *Wolbachia* into novel populations (Xi et al. 2005). This has led to two proposed mosquito control mechanisms using *Wolbachia*. First, *Wolbachia* could be used to drive a desired transgene into the mosquito population either by infecting transgenic mosquitoes with *Wolbachia* or

by creating transgenic *Wolbachia* for paratransgenesis. Alternatively, *Wolbachia* could be used directly to alter the mosquito population structure or vector competence. However, before these strategies can be employed more must be known about the interactions between the pathogen and symbiont and the effects of co-infection on the mosquito host.

Wolbachia infection alone is known to have diverse effects on host fitness. The *wMelPop* strain of *Wolbachia* is known to decrease the lifespan of both its natural host and other hosts when artificially transferred (Min and Benzer 1997, McGraw et al. 2002, McMeniman et al. 2009). This negative effect has led some authors to classify infection by this strain as pathogenic instead of symbiotic (Suh et al. 2009). Conversely, another study showed that female *Ae. albopictus* infected with their natural *Wolbachia* strains lived longer and laid more eggs than females cured of their *Wolbachia* infections (Dobson et al. 2004). Despite this evidence, other studies have found no phenotypic effect of *Wolbachia* infections on mosquitoes. For instance, when male *Ae. albopictus* are cured of their *Wolbachia* infection by tetracycline treatment there is no difference between the wild type and cured mosquitoes in lifespan or mating potential (Calvitti et al. 2009). Similarly, no negative effects on fecundity were noted in *Ae. aegypti* mosquitoes artificially transfected with *wAlbB* from *Ae. albopictus* (Xi et al. 2005).

These lifespan effects could be utilized for the purposes of vector control. For instance, because the *wMelPop* strain of *Wolbachia* can reduce mosquito lifespan significantly, the introduction of this strain of *Wolbachia* could be used to cause mosquito death before the extrinsic incubation period of an arbovirus has

passed, thereby reducing or stopping transmission (McMeniman et al. 2009). Alternatively, *Wolbachia* has been shown to reduce dengue virus infection levels and strength in *Ae. aegypti* and Chikungunya virus levels in *Ae. albopictus*, so the very presence of *Wolbachia* in mosquitoes may be enough to reduce human cases (Moreira et al. 2009b, Bian et al. 2010, Mousson et al. 2010).

While the primary vector of dengue virus, *Ae. aegypti,* is not naturally infected with *Wolbachia*, it has been infected with both the *wAlbB* strain from *Ae. albopictus* and *wMelPop* strain from *Drosophila melanogaster* by embryo microinjection and has proven to be a competent host with strong CI and a 100% maternal transmission rate (Xi et al. 2005, McMeniman et al. 2009). Because of its importance as a disease vector, *Ae. aegypti* has received considerable scientific attention towards development of a gene drive mechanism. Due to the tools available to us and the recent introduction of *Wolbachia* into this species, *Ae. aegypti* provides us a perfect opportunity to investigate the effects of dengue virus-*Wolbachia* co-infection on an important vector species. Here, we investigate how this co-infection affects the lifespan and fecundity of host mosquitoes.

Materials and Methods

Mosquito rearing: Mosquitoes of the Waco and artificially *Wolbachia* infected WB1 strains of *Ae. aegypti* were reared according to standard procedures. Eggs were hatched in double distilled water under a vacuum and larvae were transferred to pans containing 750 mL of water to grow. Larvae were maintained

at low densities (approximately 200 larvae/pan) and fed on a 6 g/100 mL liver powder and water solution ad liberum until pupation, at which point they were moved to a cup of distilled water in a large cage. After eclosion, adults were provided with cotton balls soaked in a 10% sucrose and water solution constantly, and weekly were allowed to take a blood meal from ketamine anesthetized mice. Three days after blood meals oviposition papers in water were provided for egg laying. Eggs were stored in a dark drawer until hatching.

Dengue virus-2 infections: The New Guinea C strain of dengue virus-2 was propagated in cells of the C6/36 line from *Ae. albopictus* and used to give mosquitoes an infectious blood meal according to a standard protocol. Briefly, C6/36 cells were grown in 25 cm^2 flasks in minimal essential medium (MEM) containing 10% heat inactivated FBS, 1% L-glutamine, and 1% non-essential amino acids and incubated at 32° C with 5% CO_2 until reaching 80% confluency. At that point, 0.5 mL of virus stock was added to the cells and they were incubated for seven days, giving a multiplicity of infection (MOI) of 3.5 virus particles per cell. Infected cells were then harvested using a cell scraper to remove them from the sides of the flask, collected by centrifugation, and lysed by quickly freezing and thawing them repeatedly by moving them between dry ice in 100% alcohol and a 37° C water bath. The lysed cell solution was resuspended in MEM and mixed with an equal volume of defibrinated sheep blood, and one week old mosquitoes were allowed to feed on the mixture while a water bath kept the blood at 37° C as described previously (Das et al. 2007). Other mosquitoes

were allowed to feed on a mixture of sterile MEM and defibrinated sheep blood to provide a non-infectious blood meal for comparison.

Effects of dual infection on mosquito longevity: To examine the effects of dengue and *Wolbachia* co-infection on mosquito lifespan, Waco and WB1 mosquitoes were provided an infectious or non-infectious blood meal and monitored until death. After the blood meal was provided, mosquitoes were moved into cups containing 10 male and 10 female mosquitoes. These cups were maintained as described above, with a constant source of 10% sucrose in water. Every day the number of dead mosquitoes of both sexes was recorded. Three days after the blood meal, mosquitoes were provided a small cup of water and paper for oviposition. Once per week, the mosquitoes were anesthetized with CO_2 and moved to a clean cup, and any dead bodies removed. The daily counts were used to construct Kaplan-Meier survival curves and subjected to statistical analysis for significance using a log-rank test.

Effects of dual infection on fecundity: To investigate the consequences of dual infections on mosquito fecundity, female Waco and WB1 mosquitoes were again provided with an infectious or non-infectious blood meal and allowed to lay eggs. The eggs were stored for one week at 27° C and 80% humidity. At that point, they were immersed in deionized water and allowed to hatch for two days. After hatching, the eggs were counted and scored visually as hatched or unhatched. Any larvae that hatched were allowed to develop to adulthood and the time spent

in each stage was recorded. Upon eclosion, the mosquitoes were sexed to determine the percent of eggs laid that were female.

Results

Effects of dual infection on mosquito longevity: To measure the effects of *Wolbachia* and dengue virus infection on mosquito longevity, we provided mosquitoes with blood meals with or without dengue virus, and kept track of how long they lived. Female mosquito survival data differed significantly among infection statuses ($p<0.01$, Kaplan-Meier log-rank) with females infected by dengue being shorter-lived than dengue uninfected females or those co-infected with dengue and *Wolbachia* (Figure 3.1a). There was no difference in survivorship between *Wolbachia* infected and uninfected males (Figure 3.1b).

Effects of dual infection on fecundity: In order to investigate how *Wolbachia* and dengue virus infection affect the fecundity of mosquitoes, we provided female mosquitoes with dengue infected or uninfected blood meals, allowed them to oviposit, and counted the number of eggs laid per female, the percent that hatched, the percent female, and the development time for the offspring. ANOVA revealed a significant effect of the blood meal source on the number of eggs laid per mosquito ($p<0.05$, ANOVA) (Table 3.1, Figure 3.2). Neither *Wolbachia* nor virus infection status had a significant effect on number of eggs laid per female, as revealed by the ANOVA. There were no significant effects of *Wolbachia* infection, virus infection, or blood source on the percent egg hatch

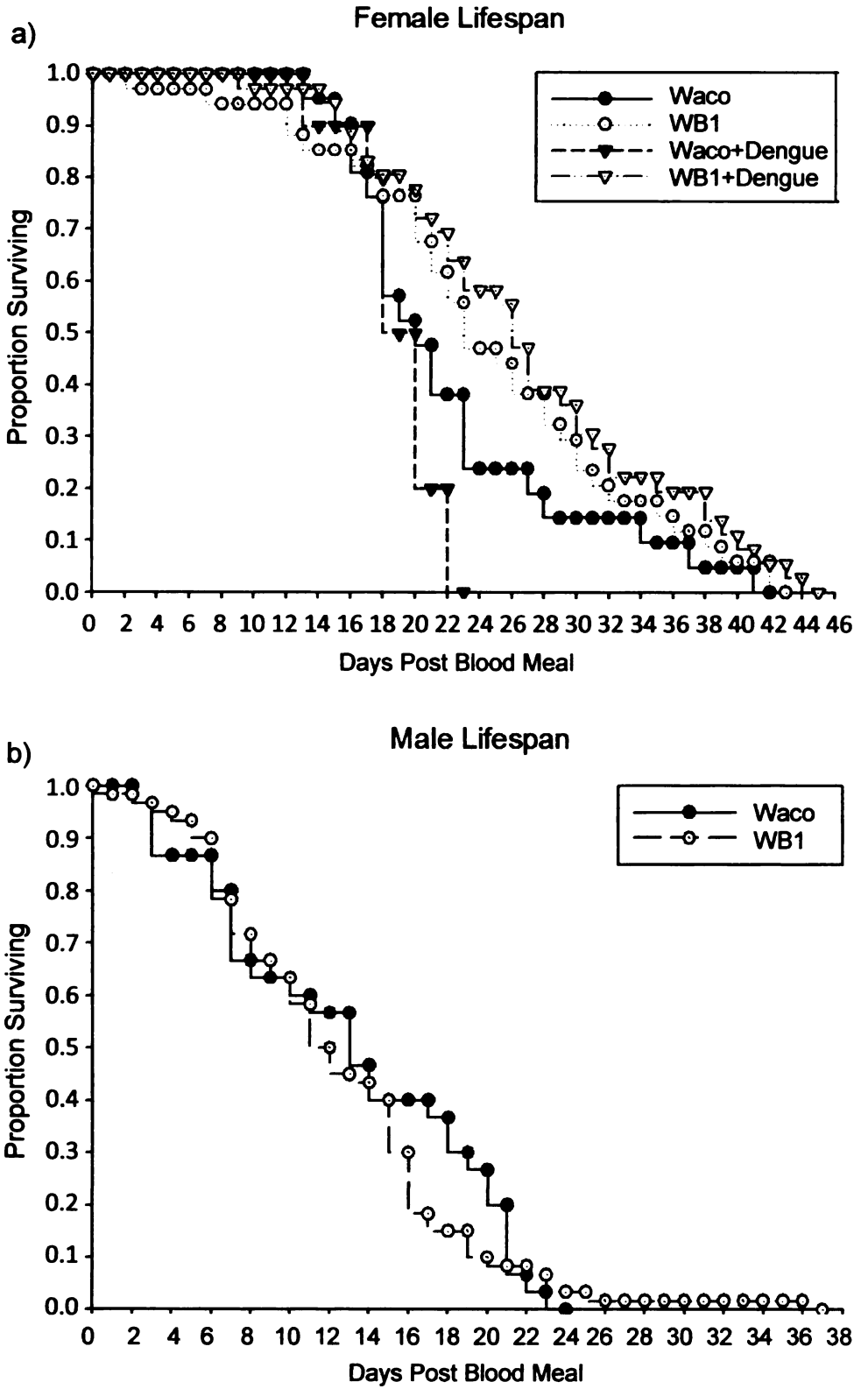

Figure 3.1: Rates of mortality for a) female and b) male *Ae. aegypti* adults with or without *Wolbachia* and dengue-2 infection.

	Eggs per female					Percent egg hatch			
	d.f.	Mean squares	F	p		d.f.	Mean squares	F	p
Wolbachia	1	1365.3	4.3345	0.08251		1	37.18	0.4933	0.5088
Virus	1	396.9	1.26	0.30454		1	129.02	1.7116	0.2387
Blood source	1	3767.1	11.9594	0.0135 *		1	3.08	0.0408	0.8465
Wolbachia x virus	1	55.2	0.1753	0.69005		1	1.32	0.0175	0.899
Wolbachia x blood source	1	1.6	0.0051	0.94516		1	265.93	3.5278	0.1094
Error	6	315				6	75.38		

Table 3.1: ANOVA testing *Wolbachia* infection, virus infection, and blood source effects for both the number of eggs laid by each female and the percent of eggs hatching.

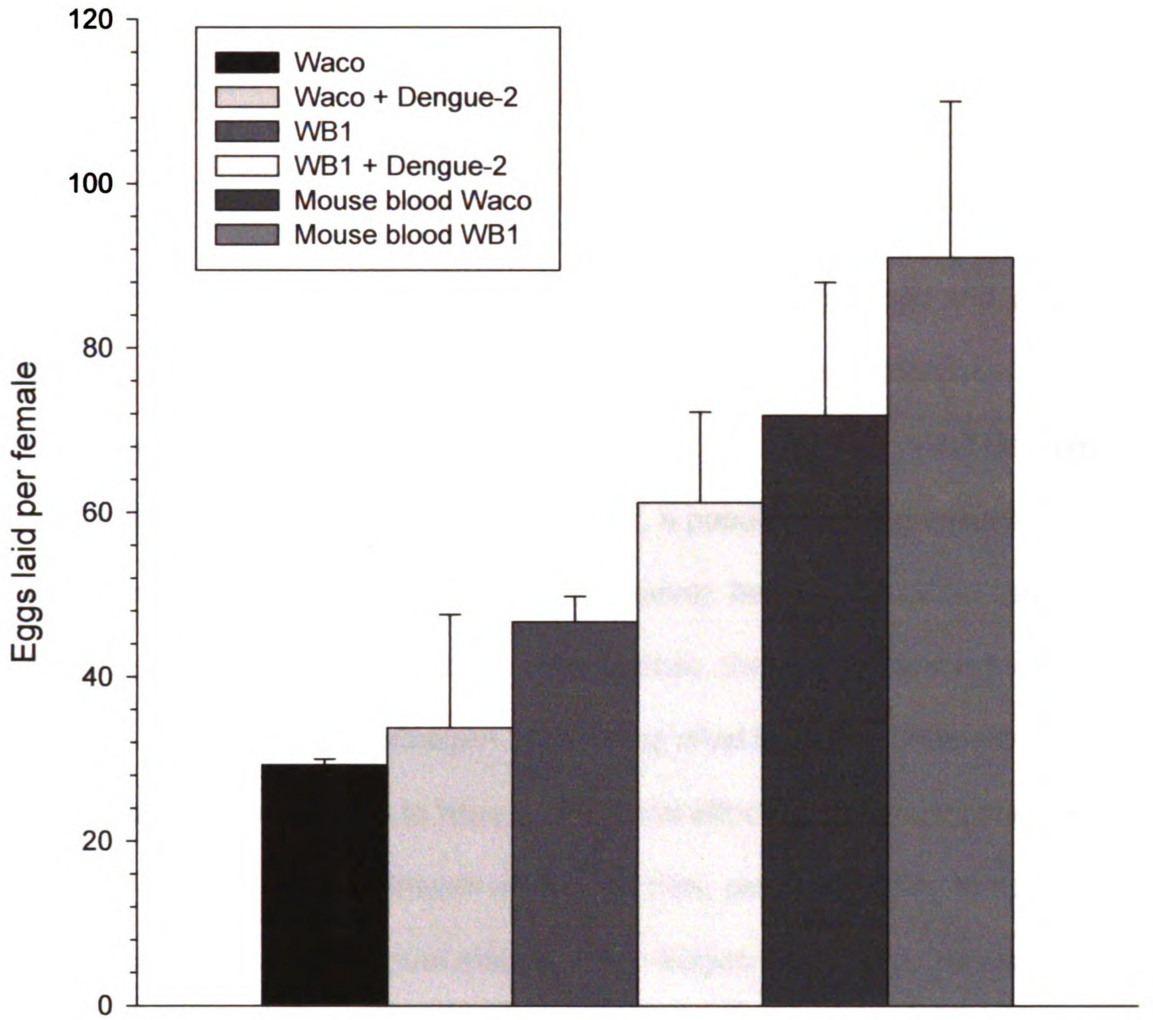

Figure 3.2: Average number of eggs laid by female *Ae. aegypti* with or without *Wolbachia* or dengue-2 infection and fed on either an artificial blood meal or mouse blood. Error bars show standard error of the mean.

(Table 3.1, Figure 3.3). We also found no difference between infection statuses in the amount of time it took for the mosquitoes to develop or the percent female (data not shown).

Discussion

Because *Ae. aegypti* is rapidly spreading through the world and, as it expands, carrying with it dengue virus and other vector-borne diseases, its control is of great public health interest. *Wolbachia* is one suggested disease control mechanism, whether as a gene driver, a population suppressant, or an immune effector itself (Bian et al. 2010). However, before *Wolbachia* can be effectively employed to fight vector-borne disease, the ways in which it interacts with both its host and the pathogen it is fighting must be better understood. *Wolbachia* has been shown to have an anti-viral effect in both mosquitoes and *Drosophila*, extending the lifespan of infected flies, possibly adding to its usefulness as a disease control mechanism (Hedges et al. 2008, Teixeira et al. 2008, Moreira et al. 2009b, Bian et al. 2010). However, some *Wolbachia* strains have also been shown to have negative effects on their host lifespan (McMeniman et al. 2009). *Ae. aegypti*, while not naturally infected with *Wolbachia,* has been stably infected with *Wolbachia* of both the *w*AlbB strain from *Ae. albopictus* and the *w*MelPop strain from *D. melanogaster*, indicating that *Wolbachia* may be useful for controlling this important disease vector (Xi et al. 2005, McMeniman et al. 2009). These infections may affect the mosquito lifespan or fecundity, which could limit the effectiveness of this control program.

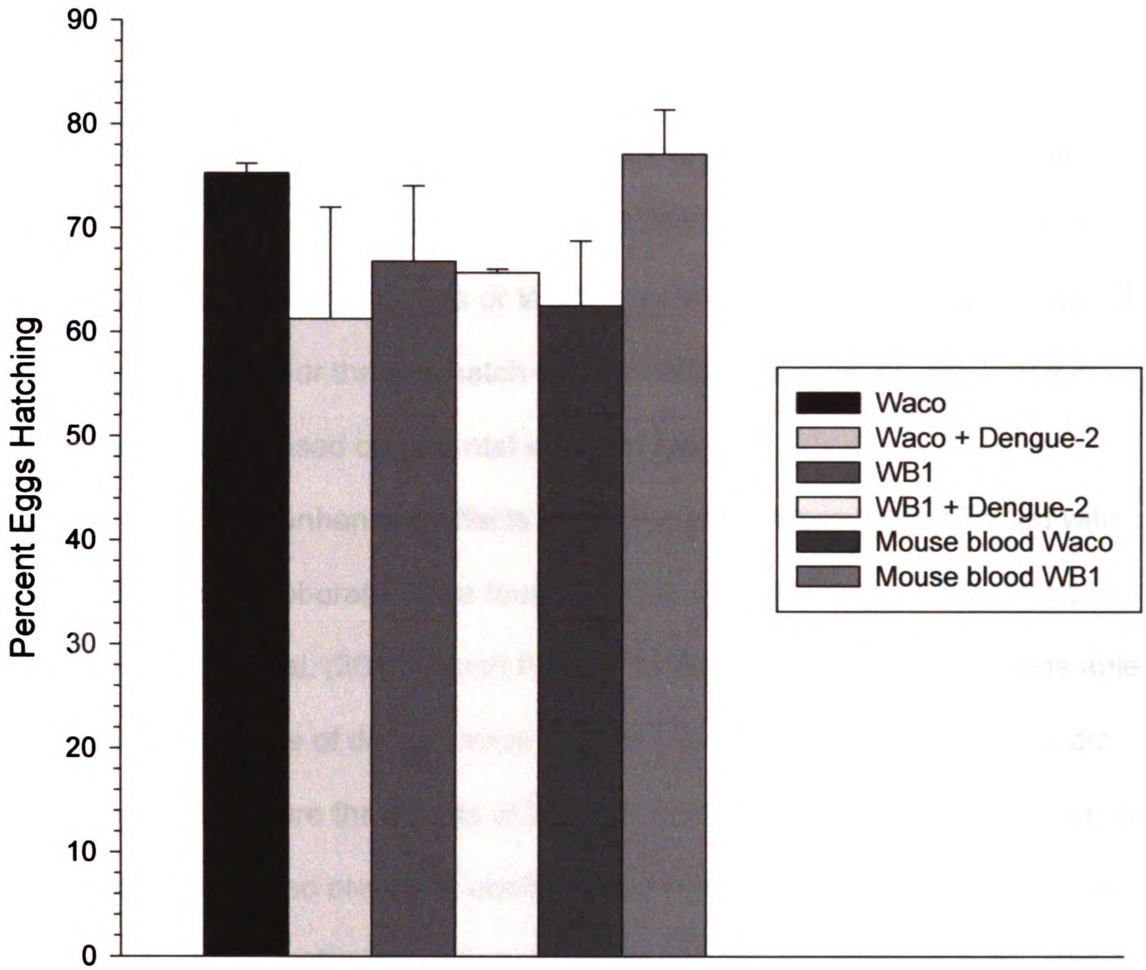

Figure 3.3: Average hatch rate of eggs laid by female *Ae. aegypti* mosquitoes with or without *Wolbachia* or dengue-2 infection and fed on either an artificial blood meal or mouse blood. Error bars show standard error of the mean.

Therefore, we measured the effects of both *Wolbachia* and dengue virus type-2 infection on mosquito lifespan, egg laying rate, and egg hatch percentage. Our results indicate that *Wolbachia* infection alone does not affect mosquito lifespan, but can remove the negative effects of dengue virus infection. We also noticed significant effects of the blood meal source on the number of eggs laid, but did not find significant effects of virus or *Wolbachia* infection on either the number of eggs laid per female or the egg hatch rate. Finally, there was no difference in development time based on parental infection type.

The lifespan-enhancing effects of *Wolbachia* in mosquitoes infected with viral pathogens corroborate those found in other studies using similar strains of *Wolbachia*. Bian et al. (2010) found that the *w*AlbB strain of *Wolbachia* was able to mediate the effects of dengue virus infection, just as we observed. They did not, however, measure the effects of *Wolbachia* infection alone on either male or female mosquitoes, so our study confirms and expands upon their results. The same strain of *Wolbachia* has also been found to have no effect on male lifespan in its natural host *Ae. albopictus*, so it is not surprising that there are no effects on male lifespan in *Ae. aegypti* (Calvitti et al. 2009). In female *Ae. albopictus*, however, *Wolbachia* infection was found to extend mosquito lifespan relative to uninfected conspecifics, while we observed no difference between *Wolbachia* infected and uninfected females (Dobson et al. 2004). This may, in part, be due to the length of association between the host and the symbiont. *Ae. albopictus* has been infected with the *Wolbachia* for a long time, and is found naturally infected in nature. *Ae. aegypti*, on the other hand, has only been infected with

Wolbachia for approximately six years since they were artificially infected (Xi et al. 2005). Therefore, we may be observing a transitional period in *Ae. aegypti* infection status in which the host is not yet well adapted to the symbiont infection. Only time will tell if the mosquitoes and *Wolbachia* evolve to a point of having more benefit to infection.

Wolbachia's ability to remove the negative effects of virus infection on mosquitoes and other insects has been well documented. Initial results in *D. melanogaster* indicated that infection with its natural *Wolbachia* flora could significantly extend the lifespan of flies infected with *Drosophila* C virus, Cricket Paralysis virus, or Flock House virus (Hedges et al. 2008). A second study corroborated these results and added resistance to Nora virus to the list (Teixeira et al. 2008). Two recent studies have shown the same effects taking place in *Ae. aegypti* infected with dengue virus (Moreira et al. 2009b, Bian et al. 2010). These studies in mosquitoes indicated that not only were the lifespans of infected mosquitoes extended, the amounts of virus accumulating within the mosquitoes was reduced. This is a positive effect for vector-borne disease efforts, because if virus titers remained high in the mosquitoes and the lifespan of virus-infected mosquitoes was lengthened, it would increase the amount of time they are able to spread the virus to new hosts. Because, however, the virus titers remain low in *Wolbachia* infected mosquitoes, the extended lifespan we observed should not contribute to the spread of disease. Instead, the extended lifespan will increase the ability of *Wolbachia* to invade the natural vector population because they will have more opportunities to lay eggs.

Along with the increased opportunities to lay eggs provided by a lengthened lifespan, we also believed that *Wolbachia* infection may increase the ability of mosquitoes to lay eggs. To that end, we tested the number of eggs laid by *Wolbachia* and dengue virus infected and uninfected females. The *w*AlbB strain of *Wolbachia* is known to increase the number of eggs laid by females of its natural host species, *Ae. albopictus*, though the same effect was not observed in *Ae. aegypti* during this study (Dobson et al. 2004). The authors of the *Ae. albopictus* study proposed that this effect would help to spread *Wolbachia* into a novel population; not only would the infected females have an advantage due to CI, they would also be laying more eggs. Despite the fact that our results were not statistically significant, the pattern is similar to that observed in *Ae. albopictus*: in all pairwise comparisons across *Wolbachia* types but within virus infection type or blood source the WB1 mosquitoes laid more eggs than their Waco counterparts. It is possible that more replicates could reveal a statistically significant effect of *Wolbachia* infection. Even if this effect is not seen at this point, it could be due to a lack of adaptation of *Ae. aegypti* to *Wolbachia* infection. When initially infected six years ago, no effect on egg production was observed at all between Waco and WB1 mosquitoes, so we may be in the midst of a change. Studies in *Drosophila* have shown that as an interaction between *Wolbachia* and its host becomes more established, the effects of *Wolbachia* infection can change (Carrington et al. 2010). This could be happening in mosquitoes too; as time goes on the mosquitoes may evolve to take advantage of their *Wolbachia* infection and lay more eggs.

Other viruses are able to affect the number of eggs laid by their hosts. For instance, *Ae. aegypti* mosquitoes infected with the *Ae. albopictus* parvovirus lay fewer eggs and have more variance in the number of eggs they lay than uninfected females (Barreau et al. 1997). This, however, is an insect-specific disease, not a human disease vectored by insects. It would be disadvantageous for a vector-borne disease to decrease the number of host eggs that are laid, as that would reduce the host population, and thus the chances of successful transmission. Also, while rare, vertical transmission of dengue has been recorded; a reduction in egg laying rates would reduce the already small chance of this happening (Hull et al. 1984, Joshi et al. 2002, Arunachalam et al. 2008). Therefore, the fact that there was no effect of virus infection on egg laying rates is not surprising.

We did, however, find a significant effect of the blood source on the egg laying numbers. Mosquitoes fed on blood directly from a live mouse laid more eggs than those fed on defibrinated sheep blood from feeders, whether or not virus was present and regardless of *Wolbachia* infection status. This likely reflects increased nutrition available from fresh blood meals as blood stored for laboratory use may break down over time, yielding less nutrition. In addition, the blood used for infective blood meals and uninfective controls are diluted with medium from the cells used to propagate the virus, so the solution the mosquitoes are feeding on is only 50% blood, 50% MEM. Thus, the blood becomes diluted, and more nutrition is lost. Because female mosquitoes depend on blood meals to get the necessary proteins to lay eggs, a diluted blood meal

will provide less protein and therefore fewer eggs can develop. This effect must be taken into account in any future studies using artificial blood meals to infect their mosquitoes with any vector-borne disease.

That we did not see any difference in egg hatch rates or development time based on parental infection status is not surprising. All of the matings we tested were compatible, so there should have been no effect of CI on the hatch rates. In a previous study, *Ae. albopictus* females infected with *Wolbachia* did have higher hatch rates than their uninfected conspecifics; this again may indicate an effect due to long association between the *Wolbachia* and host that is not yet seen in *Ae. aegypti* (Dobson et al. 2004). Dengue virus infection has been shown to have an effect on larval duration in one past study, but their study was based on the dengue-3 strain, while ours worked with the dengue-2 strain (Joshi et al. 2002).

In general, our results indicate that *Wolbachia* infection is largely beneficial for the mosquito *Ae. aegypti*. No negative effects of *Wolbachia* infection were observed, and some positive effects were seen. The fact that *Wolbachia*-infected mosquitoes live longer after infection with dengue virus type-2 indicates that *Wolbachia* can protect the mosquitoes from the negative effects of this virus and allow them more opportunities to lay eggs than they would otherwise have. Also, the trend, though not statistically significant, of *Wolbachia*-infected females laying more eggs than their uninfected counterparts indicates that *Wolbachia* infection is moving toward a point of greater host benefit, again allowing the infected mosquitoes to lay more eggs and increasing their fitness

advantage. Virus infection was seen to negatively affect mosquito lifespan, but did not affect the egg laying rate at all, and none of the treatments we applied had any effect on the egg hatch percentages or development time of our mosquitoes. The significant difference in the number of eggs laid by females fed on live mouse blood versus stored blood is important to consider for laboratory studies, but will not have much effect on natural systems where the mosquitoes are always feeding off of live hosts. Taken together, these results indicate that *Wolbachia* will be able to effectively invade *Ae. aegypti* populations due to their increased fitness coupled with the effects of CI. This makes *Wolbachia* all the more appealing as a vector control tool, while making it even more curious that this mosquito species is not naturally infected with *Wolbachia*. While the interactions between symbionts, pathogens, and hosts are still not fully elucidated, this study shows that this system is sustainable in the laboratory and that *Wolbachia* should continue to be studied for potential use in controlling dengue virus.

CHAPTER 4

Mosquito, *Wolbachia*, dengue interactions: Conclusions and future directions

Conclusions

Before *Wolbachia* can be employed as an effective vector control mechanism, the factors that affect its spread and persistence must be more clearly understood, as must the effects of *Wolbachia* infection on its hosts and the pathogens it targets. In this study, we have addressed some of these issues by investigating how the mosquito immune system is able to affect *Wolbachia* densities and how infection with *Wolbachia* and dengue virus can affect the mosquito host. We observed a significant effect of the mosquito Imd pathway and gram-negative bacterial infection on *Wolbachia* densities. An increase in both the Toll and Imd pathways at the same time causes an increase in ovarian *Wolbachia,* while a decrease in the expression of Imd pathway genes causes a decrease in *Wolbachia* densities of mosquito ovaries. Similarly, increasing the amount of gram negative bacteria infecting the mosquitoes causes a decrease in the density of *Wolbachia* in mosquito ovaries. This pattern indicates that the effects of the mosquito immune system on *Wolbachia* are not direct, but act indirectly through modulating densities of gram negative bacteria. Because the Imd pathway specifically targets gram negative bacteria, decreasing the expression of Imd pathway genes could lead to an increase in the numbers of gram negative bacteria, after which competition with *Wolbachia* could lead to a decrease in the symbiont.

We also saw that infection with *Wolbachia* can increase the lifespan of mosquitoes co-infected with dengue virus type-2. This effect has been observed in other mosquito-pathogen interactions and in *Drosophila* (Hedges et al. 2008, Teixeira et al. 2008, Moreira et al. 2009b, Bian et al. 2010). There was not any effect of *Wolbachia* infection alone on either male or female lifespans, nor was there an effect of any infection type on the percent of eggs that hatch. While we observed no difference in the number of eggs laid by females infected with *Wolbachia*, dengue, both, or neither, we did see that there was a significant effect of the type of blood used to feed the mosquitoes. Those mosquitoes fed on blood from live mice laid more eggs than those fed with artificial blood mixed with MEM, regardless of infection status. There was also a pattern of increased egg laying by *Wolbachia*-infected mosquitoes compared to their *Wolbachia* free counterparts, though the effect was not statistically significant. This group of effects is similar to those seen in *Ae. albopictus* mosquitoes infected by the same strain of *Wolbachia* (Dobson et al. 2004). This increase in the lifespan of mosquitoes infected by both a virus and *Wolbachia* may indicate another advantage *Wolbachia* confers on its hosts, increasing the probability of its spread. Similarly, allowing infected females to lay more eggs than uninfected females would increase the fitness advantage afforded the infected females and therefore the spread of *Wolbachia*, and any genes associated with it, into the population.

Future Directions

While these results show a great deal of promise for using endosymbionts in the future battle against vector-borne disease, many issues still need to be resolved. The mode of action, be it competition, immune activation, or some other system entirely, needs to be elucidated before it can be fully utilized. Without a more complete knowledge of how the symbionts and pathogens are interacting with each other and their mutual host, it is entirely possible that any attempts to use these strategies could end in failure. For instance, if infection with the pathogen will simply lead to the loss of the symbiont from the host, the symbiont will cease to be an effective control mechanism. On the other hand, if the symbionts are able to clear the pathogen without the need for the creation of transgenic mosquitoes, it will make the entire process simpler; there will be no need to get approval for the release of transgenic insects and any scientific barriers to doing so will be irrelevant. A fuller understanding of how these organisms interact will be necessary before any plan involving them can be implemented, because convincing people to allow the release of organisms artificially infected with a pathogen may be a difficult battle.

Were we to have a full understanding of how the symbionts and pathogens interact, we would still be unable to use these systems effectively today. While the recent infection of *Ae. aegypti* with various types of *Wolbachia* has been a major step in this direction, many other important vector species have yet to be successfully infected (Xi et al. 2005, McMeniman et al. 2009). Most notably, no species of *Anopheles* mosquitoes are naturally infected with

Wolbachia and attempts to artificially transfect them have been unsuccessful (Rasgon et al. 2006, Jin et al. 2009). Despite the fact that *Wolbachia* can become established in both *Anopheles* spp. cells and adults when directly injected, as yet there have been no stable infections created. This may be the result of any number of factors, including the innate immune system of the mosquitoes or competition with natural microflora, but it remains an important barrier to their use. Because *Anopheles* spp. mosquitoes are the main vectors of human malaria throughout the world, if they cannot be infected with *Wolbachia*, then *Wolbachia*-based intervention strategies will be ineffective against malaria. Malaria caused nearly one million deaths in 2006, so any vector control strategy that is not effective against malaria will not be a complete success (WHO 2010).

In addition to attempts to introduce *Wolbachia* into new host species, efforts continue to introduce other symbionts into new hosts and to create transgenic *Wolbachia*. There are a number of other symbionts which infect insects, such as *Wigglesworthia* and *Sodalis* in tsetse flies, *Buchnera* in aphids, and many others (Shigenobu et al. 2000, Rio et al. 2006, Moran et al. 2008). While *Wolbachia* is the most well studied of these symbionts, the others may be as important to the immunity of their hosts. Studying them will only expand our knowledge in this area. It is possible that by transferring these infections to new hosts, other tools to fight vector-borne disease may be developed. At the same time, given that *Wolbachia* can spread into the population it infects, focusing on it as a control mechanism is a good idea. However, if it proves that *Wolbachia* alone cannot prevent pathogen infections, it will be necessary to use other

methods in conjunction with *Wolbachia*. One possibility is to use *Wolbachia* to give transgenic mosquitoes an advantage over wild-type mosquitoes, thus spreading the desired transgenes into the populations (Sinkins and Godfray 2004). Another option would be to genetically modify the *Wolbachia* itself to avoid the possibility of separation of the transgene and the gene driver. Despite the fact that other *Rickettsia*-like bacteria have been transformed, there have not yet been any successful transformations of *Wolbachia* (Baldridge et al. 2005).

Alternatively, if symbiont infection proves inadequate to prevent vector-borne disease and transgenic insects are to be employed, it may be possible to create transgenic insects which spread into the population without the need for *Wolbachia* as a gene driver. Numerous other gene drive systems have been proposed, including transposable elements, underdominance, meiotic drive, and homing endonucleases, but most of these techniques have not been proven (Sinkins and Gould 2006). It is also possible to induce the effects of cytoplasmic incompatibility in insects without the need for bacteria by engineering a modification and rescue system similar to that proposed for *Wolbachia* right into the insect genome (Chen et al. 2007). While Chen et al. (2007) have proven that microRNA based silencing of genes can lead to CI-like effects in *Drosophila*, their results need to be extended to important vector species. A system such as this would not require any infection by symbionts and would limit the possibility that the transgenes could become unlinked from the gene drive mechanism, making it a good candidate for use in vector control. Also, as transgenic *Anopheles* mosquitoes have been successfully created, this strategy could be employed in

the malaria system where *Wolbachia*-based strategies are not yet possible (Catteruccia et al. 2000).

While the use of symbionts to control vector-borne disease is still a relatively young field, it shows great promise. The fact that infection of insects with symbionts such as the bacteria *Wolbachia* can lead to a reduction in vectorial capacity of those insects indicates that it may be possible to use these symbionts to reduce the burden of vector-borne disease throughout the world. While the set of interactions between pathogens, symbionts and hosts has not been fully realized, and the mechanisms behind those interactions are largely unknown, past results indicate that infection with one microorganism can greatly impact future infection by others. Whether these interactions are based on immunity or competition, and whether the vector control strategy is based on driving novel genes into a population or simply changing the population levels and age structure, there is great potential here for reducing or removing vector-borne diseases from circulation. Because diseases such as dengue fever and malaria cause such high rates of morbidity and mortality throughout the world, this line of investigation merits further research, as it could save millions of lives.

Appendix 1.1

Record of Deposition of Voucher Specimens*

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2010-03

Title of thesis or dissertation (or other research projects):

Three Way Interactions Between *Wolbachia*, Dengue Virus, and their Host *Aedes aegypti*

Museum(s) where deposited and abbreviations for table on following sheets:

Entomology Museum, Michigan State University (MSU)

Other Museums:

Investigator's Name(s) (typed)

Andrew Pike

Date 12 May 2010

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America.
Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation.
Museum(s) files.
Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

Appendix 1.2

Voucher Specimen Data

Page 1 of 1 Pages

Species or other taxon	Label data for specimens collected or used and deposited	Number of: Eggs	Larvae	Nymphs	Pupae	Adults ♀	Adults ♂	Other	Museum where deposited
Aedes aegypti (L.) strain: Waco	Michigan; Ingham Co. MSU 306 Giltner Hall 10-May-2010					5	5		MSU
Aedes aegypti (L.) strain: WB1	Michigan; Ingham Co. MSU 306 Giltner Hall 10-May-2010					5	5		MSU
Aedes aegypti (L.) strain: UgalB	Michigan; Ingham Co. MSU 306 Giltner Hall 10-May-2010					5	5		MSU
Aedes aegypti (L.) strain: Rel1B	Michigan; Ingham Co. MSU 306 Giltner Hall 10-May-2010					5	5		MSU
Aedes aegypti (L.) strain: Rel2B	Michigan; Ingham Co. MSU 306 Giltner Hall 10-May-2010					5	5		MSU

(Use additional sheets if necessary)

Investigator's Name(s) (typed)

Andrew Pike

Date 5/12/2010

Voucher No. 2010-03

Received the above listed specimens fc deposit in the Michigan State University Entomology Museum.

Curator 5/12/2010

Date

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