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# CHARACTERIZATION OF *WOLBACHIA* AND ITS INTERACTION IN HOST MOSQUITOES

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ABSTRACT OF DISSERTATION

Eunho Suh

The Graduate School

University of Kentucky

2011

# CHARACTERIZATION OF *WOLBACHIA* AND ITS INTERACTION IN HOST MOSQUITOES

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ABSTRACT OF DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Eunho Suh

Lexington, Kentucky

Director: Dr. Stephen L. Dobson, Professor of Entomology

Lexington, Kentucky

2011

ABSTRACT OF DISSERTATION

# CHARACTERIZATION OF *WOLBACHIA* AND ITS INTERACTION IN HOST MOSQUITOES

*Wolbachia* are maternally inherited, obligate, intracellular bacteria inducing a form of sterility known as cytoplasmic incompatibility. *Wolbachia* based strategies have been proposed for the control of disease vectors. One example is to use a population replacement strategy to drive into natural population a novel *Wolbachia* that modifies the age structure of a vector population, reducing disease transmission.

In this research, the effects of a life-shortening stain of *Wolbachia* (*popcorn Wolbachia*) are transferred into the mosquitoes *Aedes albopictus* (Chapter Two and Three) and *A. aegypti* (Chapter Four and Five). In Chapter Two, the *Wolbachia* symbiosis significantly reduced fecundity and egg hatches in *A. albopictus*, with *Wolbachia* being highly pathogenic in this mosquito species. In Chapter Three, the relationship between *popcorn Wolbachia* and its host (in a triple-infected mosquito strain) varied with the mosquito diet. Feeding on mouse blood was associated with the loss of infection, whereas the infection was maintained in human blood-fed mosquito lines. Egg viability of triple infected mosquito was reduced only with mouse blood.

In Chapter Four, the reduced competitiveness (e.g., low survival and increased developmental time) of infected *A. aegypti* immatures was associated with *popcorn Wolbachia*, relative to uninfected individuals in low food condition. In Chapter Five, the decreased survival of immature *A. aegypti* was associated with *popcorn Wolbachia* in the presence of potential predators (i.e., older *A. aegypti* or *A. albopictus* larvae). Using a novel behavioral assay, a delayed larval reaction to light avoidance was observed to be associated with the infection, suggesting *Wolbachia* effects on immature host behaviors.

In Chapter Six, *popcorn Wolbachia* and *w*AlbB infected *A. aegypti* showed similar reproduction potential. No reduced level of CI or mating competitiveness was observed in *w*AlbB infected males. The results suggest the *w*AlbB infection in *A. aegypti* can be an additional agent for *Wolbachia*-based control strategies.

In Chapter Seven, a filtering system using commercially available sieves was able to separate immature mosquitoes from water, preventing escape of mosquitoes. In Chapter Eight, an inexpensive artificial blood feeding was designed for feeding multiple mosquito cages. The results support the use of these tools to facilitate mass rearing of mosquitoes.

KEYWORDS: cytoplasmic incompatibility, Dengue, arbovirus, fitness cost, insect behavior

Eunho Suh

Student's Signature

July 25, 2011

Date

# CHARACTERIZATION OF *WOLBACHIA* AND ITS INTERACTION IN HOST MOSQUITOES

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DISSERTATION

Eunho Suh

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Dedicated to my wife, Sunhee Yun, and my daughter, Yuna Suh

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# Chapter One

## Introduction

Among the most prevalent organisms on earth, the role of insects in nature is essential. They occur almost everywhere and play important roles, ranging from beneficial pollinators of plants to more detrimental roles as pests and disease vectors of crops, livestock and humans. Particularly, insect-borne diseases have become an enormous threat to the global health of humans. Malaria alone results in an estimate of over 220 million cases and up to one million deaths per year (WHO 2010). Mosquito borne diseases are arguably the most important diseases with at least half of the world population at the risk of infection. The incidence and potential risk of mosquito borne disease is increasing annually due to urbanization of rural areas, global transportation of goods and human travelers (Gould and Solomon 2008, Adams and Kapan 2009, Chen and Wilson 2010). Moreover, sustained vector control methods have not always been successful. For example, the repeated use of insecticides has often caused vector resistance resulting in reduced effectiveness of the insecticide use. Furthermore, increasing concerns about the negative impacts of insecticides on the environment have also prompted interest in the development of novel and environmentally friendly control strategies.

*Wolbachia pipientis* is a maternally inherited, obligate, intracellular bacterium in the order of Rickettsiales from the group of alpha-Proteobacteria (Werren 1997, Werren et al. 2008). A number of studies have detected *Wolbachia* in multiple species of invertebrates, including insects, spiders, scorpions, mites, springtails, terrestrial isopods

and filarial nematodes (Werren 1997, Zhou et al. 1998, Jeyaprakash and Hoy 2000, Hilgenboecker et al. 2008, Werren et al. 2008) since *Wolbachia* was first described in the mosquito *Culex pipiens* (Hertig and Wolbach 1924). A recent study suggested *Wolbachia* is one of the most widely spread bacterium on earth, infecting over 65% of insect species (Hilgenboecker et al. 2008).

The pandemic distribution of *Wolbachia* in arthropods can be attributed to its ability to manipulate host reproduction (Werren et al. 2008). In most arthropods, *Wolbachia* is frequently observed as a reproductive parasite, capable of inducing feminization, parthenogenesis, male-killing, or cytoplasmic incompatibility (CI) (O'Neill et al. 1997, Werren 1997, Werren et al. 2008). For example, feminization in Hemiptera, Isopoda, and Lepidoptera can result in genetic males that develop as females. In Acari, Hymenoptera, and Thysanoptera, parthenogenesis induction enables a female to produce only females. *Wolbachia* can kill males to support the survival of infected females, which is often observed in Coleoptera, Lepidoptera, Diptera and Pseudoscorpiones.

CI is the most common and well-studied phenotype of *Wolbachia*, which is observed in several arachnids isopods and insect orders (Werren et al. 2008). CI is a form of sterility resulting from an interaction of two distinct components: a *Wolbachia*-induced "modification" in sperm and a "rescue" of this modification in embryos that include the same *Wolbachia* infection type. Sperm from *Wolbachia*-infected males is incompatible with eggs from females that do not harbor the same *Wolbachia* type. Thus, if a CI-inducing *Wolbachia* "modifies" the sperm of infected males, embryogenesis in the egg is disrupted unless the egg is "rescued" by the same *Wolbachia* strain. Although an exact molecular mechanism of CI is unknown, common cytological observation from

several taxa show that defects in early embryonic mitosis result from the disruption of the cell cycle, causing asynchronous development of male and female pronuclei (Reed and Werren 1995, Lassy and Karr 1996, Tram and Sullivan 2002). As *Wolbachia* is exclusively inherited by females, an increased relative fitness of infected females resulting from CI promotes the spread of *Wolbachia* through a population (Turelli and Hoffmann 1991, Dobson et al. 2001, 2002b, Xi et al. 2005a)

Unidirectional CI can occur in crosses between infected males and uninfected females (Fig. 1A). Reduced reproduction of uninfected females can result from CI which causes sterility in uninfected females when mated with infected males. In contrast, infected females are compatible with both infected and uninfected males, thus resulting in increased relative reproduction of infected individuals in a panmictic population. Consequently, infected individuals are capable of invading uninfected population resulting in the fixation of *Wolbachia* in the population.

*Wolbachia* induced CI can impact the reproductive outcome of mating partners if a population is infected with multiple infection types. When multiple *Wolbachia* infection types exist in a population, CI shows the additive pattern; males are only compatible with females that are infected with same *Wolbachia* type(s) observed in the males (Fig. 1B). Similar to the unidirectional CI caused by a single infection, the CI pattern by superinfected individuals can result in the replacement of single infected individuals. Bidirectional CI can be induced when two different *Wolbachia* types exist in different individuals within a population. "Modified" sperms cannot be "rescued" unless common *Wolbachia* strains exist between mating partners, resulting in sterility of females that mated with males with different *Wolbachia* infection type (Fig 1C).

*Wolbachia* induced CI has attracted considerable interest as a potential tool for novel vector control strategies. Such strategies include: (1) population replacement strategy to deliver desired phenotypes into natural populations utilizing the reproductive advantage conferred by *Wolbachia*-induced CI; (2) population suppression strategy to induce sterility by using *Wolbachia*-induced CI and reduce and/or eliminate vector population.

**Population replacement**

**Transgenic *Wolbachia* as a gene driving vehicle**. Advances in developing transgenic insects have suggested specific strategies of utilizing population replacement in vector controls. For example, refractoriness of disease induced by transgenes in genetically modified insects can be delivered into the vector population via population replacement in order to reduce pathogen transmission (Olson et al. 1996, Ito et al. 2002, Franz et al. 2006). However, such population replacement strategies require gene drive systems that will allow delivering desired transgenes into the target population. For example, *Wolbachia* was observed to spread rapidly through host populations due to a reproductive advantage afforded by CI in laboratory and field conditions (Turelli and Hoffmann 1991, Hoffmann and Turelli 1997, Xi et al. 2005a). Thus, if an anti-pathogenic transgene is linked to a *Wolbachia* infection, the *Wolbachia* is expected to spread into a target population resulting in reduced disease transmission by the expression of the transgene. One possible strategy to link *Wolbachia* and a transgene is via genetic transformation of *Wolbachia* that express anti-pathogenicity in vector hosts. Advantages using this strategy are attributed to: 1) the wide distribution of *Wolbachia* in host tissues (Dobson et al.

2002c, Chen et al. 2005, Sinkins and Gould 2006) that would allow development of tissue specific expression of the transgenes, 2) the potential for artificial transfer of transformed *Wolbachia* into new hosts, and 3) the effective gene driving system that would require less efforts to initiate population replacement (i.e., smaller population size of transformed *Wolbachia* infected to be released) due to maternal inheritance of *Wolbachia*. Although a successful transformation of *Wolbachia* has not been reported to date, several strategies are suggested and being developed for the transformation of *Wolbachia* (Chauvatcharin et al. 2006, Kurz et al. 2009).

**Natural *Wolbachia* infections and population replacement.** Min and Benzer (1997) have discovered a variant strain of *Wolbachia* that reduces a half of life span in *Drosophila melanogaster*. Unlike other strains of *Wolbachia*, the life-shortening *Wolbachia* caused high mortality of adult flies. Electro-microscopic observation revealed the host mortality was due to the uncontrolled replication of the *Wolbachia* resulting in rupture of host cells. The over-proliferating *Wolbachia* strain was named as *popcorn Wolbachia* due to the resemblance to a microwave popcorn. The virulence of *popcorn Wolbachia* was more pronounced in older flies (Min and Benzer 1997), which suggested an idea of utilizing the life-shortening *Wolbachia* in developing a novel vector control strategy (Brownstin et al. 2003, Reynolds et al. 2003). A female mosquito must survive an extrinsic incubation period (EIP) to transmit pathogens which replicate and develop to be infectious within the mosquito during EIP. Therefore, a *Wolbachia*-induced shift in the population age structure toward younger females is expected to reduce pathogen transmission by decreasing frequency of older females that are responsible for the majority of disease transmission.

As the host's fitness directly affects the ability of *Wolbachia* to spread into population, the impact of *Wolbachia* on host fitness parameters need to be closely examined. If the infection imposes an infection cost to the reproductive fitness of its host, the rate of *Wolbachia* spread is reduced and consequently the numbers of required initial releases should be increased (Turelli 2010). Although an example of *Wolbachia* in *A. aegypti* shows that phenotype of *Wolbachia* may be modified via a pre-acclimation in closely related species (e.g., *A. albopictus*) (McMeniman et al. 2008), the phenotype is generally uncontrollable upon a direct transfection. In particular, *Wolbachia* impacts on host fitness can be diverse, presenting a moderate to extreme physiological cost (Hoffmann et al. 1990, Fleury et al. 2000, Perrot-Minnot et al. 2002, Huigens et al. 2004, Rigaud and Moreau 2004, Duron et al. 2006, Islam and Dobson 2006, Suh et al. 2009, McMeniman and O'Neill 2010), an absence of cost (Harcombe and Hoffmann 2004, Montenegro et al. 2006) and benefits (Bandi et al. 1999, Hoerauf et al. 1999, Vavre et al. 1999, Dedeine et al. 2001, Dobson et al. 2002b, Fry et al. 2004, Hedges et al. 2008, Teixeira et al. 2008, Brownlie et al. 2009), which can directly affect host population dynamics. Thus, the model studies that reflect the observed effects of *Wolbachia* will help increasing the probability of success for the proposed strategy.

*Wolbachia* are frequently detected in several mosquito species including *Culex pipiens* (Yen and Barr 1973), *C. quinquefasciatus*, *Aedes fluviatilis* (Moreira et al. 2009b), and *A. albopictus* (Sinkins et al. 1995). However, the major vectors for dengue fever (*A. aegypti*) and malaria (*Anopheles* spp.) are not naturally infected with *Wolbachia*. Thus, a *Wolbachia* based strategy may require the introduction of *Wolbachia* into the target species. With development in transfer techniques of *Wolbachia* such as embryonic

microinjection, several *Wolbachia* strains have been successfully established in novel hosts across species (Xi et al. 2005b, Xi et al. 2006, McMeniman et al. 2009, Calvitti et al. 2010, Fu et al. 2010). However, transfection of *Wolbachia* is not always successful for unknown reasons, resulting in no maternal transmission of *Wolbachia* to progeny, as observed in the example of *Anopheles gambiae* transfected with the life-shortening *Wolbachia* (Jin et al. 2009). Success in the *Wolbachia* transfer may be dependent on the genetic closeness of two host species where the transfection occurs, yet further studies need to identify the underlying mechanism of *Wolbachia* colonization in reproductive tissues that enables further stability of *Wolbachia* infection through maternal inheritance.

Recently, a life-shortening infection was introduced into the dengue vector *Aedes aegypti* (McMeniman et al. 2009). Prior to the transfer of the *Wolbachia* to *A. aegypti*, *w*MelPop strain from *Drosophila melanogaster* has been maintained for three years in the cultured cell line of *A. albopictus* to create *w*MelPop-CLA (cell-line-adapted) strain of *Wolbachia* (McMeniman et al. 2008). The *w*MelPop-CLA has consistently shown strong life-shortening effect to reduce half of life span in *A. aegypti* (McMeniman et al. 2009). The infection also showed high rates of maternal inheritance and complete CI, which supports the candidacy of this transfected strain of mosquito for the population replacement strategy. However, the *w*MelPop-CLA was responsible for reduced egg viability over time that can significantly decrease the rate of *Wolbachia* spread (McMeniman and O'Neill 2010). The model studies that were designed to resemble more field-like conditions (e.g., overlapping generation) showed a significant increase in initial release number to reach a population replacement threshold due to the observed cost of the *Wolbachia* infection on host fitness (Turelli 2010, Yeap et al. 2011). Thus, the results

also suggested a further examination on other life-stages of a mosquito such as the immature stage that can have great impacts on the population dynamics of mosquitoes and *Wolbachia*.

In recent studies, several *Wolbachia* strains have been found to interfere with viruses in *Drosophila* and human pathogens in mosquitoes. Naturally occurring *Wolbachia* were responsible for the increased survival of hosts, protecting them from viruses such as *Drosophila* C (DCV), Cricket Paralysis, Nora virus, and Flock House virus (FHV) (Hedges et al. 2008, Teixeira et al. 2008), West Nile virus (Glaser and Meola 2010), and fungus *Beauveria bassiana* (Panteleev et al. 2007). Similar results have been observed in artificially transfected mosquitoes that *Wolbachia* interfered with pathogens including nematodes and bacteria (Kambris et al. 2009), viruses such as Dengue and Chikungunya (Moreira et al. 2009b, Bian et al. 2010) in addition to the avian and rodent malaria parasites *Plasmodium gallinaceum* (Moreira et al. 2009b) and *P. berghei* (Kambris et al. 2010). Although an exact mechanism for the interference of *Wolbachia* on pathogens has not been demonstrated, a common hypothesis for the observed results is that the pathogen interference in the insect is associated with the induction of antimicrobial peptides and pre-activation of the innate immune response (Kambris et al. 2009, Moreira et al. 2009b, Kambris et al. 2010). As a means for sustainable controls, vector populations could be replaced with individuals that are infected with *Wolbachia* strains that confer resistance to pathogens, thus ultimately reducing disease transmission. However, upregulation of immune response system was only observed in artificial association between *Wolbachia* and mosquitoes (Kambris et al. 2009, Moreira et al. 2009b, Kambris et al. 2010). Therefore, additional studies will be

useful to better understand the mechanism of interaction between *Wolbachia*, pathogens and hosts in order to increase applicability and sustainability in utilizing *Wolbachia* infection in the applied strategies.

**Incompatible insect technique (IIT)**

*Wolbachia*-based incompatible insect technique (IIT) strategy aims to artificially sustain female sterility by repeated releases of cytoplasmically incompatible males. The strategy is analogous to the sterile insect technique (SIT), but SIT males often lose fitness due to the technology used to generate sterile males (e.g., irradiation and chemosterilization) (Alphey 2002, Benedict and Robinson 2003). Incompatible males are generated via establishing *Wolbachia* infections in insect hosts, thus assessing the effects of *Wolbachia* infection on host fitness can be crucial for the success of IIT strategy.

Important hypotheses for the success of IIT strategy include: 1) *Wolbachia* infections display high level of CI against wild type population (i.e., low or absent egg hatch resulting from incompatible matings); 2) Maternal inheritance frequency of *Wolbachia* is high, and males sustain incompatibility induced by *Wolbachia*; 3) cost of infection on host fitness is low and male mating competitiveness are comparable to wild type males; 4) negative side effects in the ecosystem due to the release of males is slight. Since the strategy requires mass releases of *Wolbachia* infected males, developing strategies for effective mass rearing of mosquitoes are essential including accurate separation of males from females. Although the previously suggested systems provides high fidelity in male separation (Focks 1980), accidental release of *Wolbachia* infected females can potentially result in population replacement of target vectors with infected

individuals (Dobson et al. 2002a, Dobson et al. 2002b). Several strategies to prevent the escapes of infected females have been suggested such as applying irradiation treatment to sterilize the potential females among separated males (Shahid and Curtis 1987, Brelsfoard et al. 2009) or developing genetic sexing techniques utilizing transgenic insects (see Nolan et al. 2011 for review). Studies such as examining vector competence for the infected females (including irradiated females) should further reduce potential risk in applying IIT.

**Future studies**

Insect control using *Wolbachia* is promising, as the release of *Wolbachia* infected individuals are ongoing as part of a population replacement field trial of controlling Dengue in Australia (Iturbe-Ormaetxe et al. 2011). Further work should focus on the understanding of *Wolbachia* infection dynamics in the field. Furthermore, studies in the role of biotic and abiotic factors on *Wolbachia* infection dynamics will provide useful information for better designing the release strategy on the target study areas. In applied aspect, interactions between pathogens, *Wolbachia* and vector hosts should be further studied to increase sustainability of the strategy and prevent negative outcome of the population replacement.

A series of transfection experiments showed successful establishment of novel *Wolbachia* infections in *Aedes* mosquitoes (Xi et al. 2005a, McMeniman et al. 2009, Fu et al. 2010). However, the phenotypic results of *Wolbachia* are difficult to predict or stable transfection is sometimes impossible. Thus, studies on the interaction between *Wolbachia* and host should provide further information to facilitate artificial transfer of

*Wolbachia*. Specifically, studies on the underlying molecular mechanism of host immune response to a *Wolbachia* infection will help understanding intracellular interaction of *Wolbachia* and host cells.

**Objectives**

My dissertation describes several *Wolbachia* based strategies for the goal of controlling disease vectors. Particularly, the strategies utilize life-shortening *Wolbachia* that is suggested to invade and modify the age structure of vector population. Thus, *Wolbachia* infection is characterized to examine the effects of *Wolbachia* on the fitness of novel hosts, which will impact *Wolbachia* infection dynamics. Specific aims are: 1) introduce and characterize life shortening *Wolbachia* in *A. albopictus*, 2) introduce the life-shortening *Wolbachia* into wild type *A. albopictus* and characterize triple *Wolbachia* infection in *A. albopictus*, 3) examine the impacts of life-shortening *Wolbachia* on different life stages of *A. aegypti*, including eggs and larval stage, and additionally male mating competitiveness of infected males, 4) understand the impacts of *Wolbachia* infection on larval interactions and behaviors of *A. aegypti*, 5) characterize *w*AlbB strain of *Wolbachia* infection in *A. aegypti* comparing with life-shortening *Wolbachia* for potential agent of population replacement or suppression strategy, 6) develop tools to facilitate mass rearing of mosquitoes in a laboratory setting.

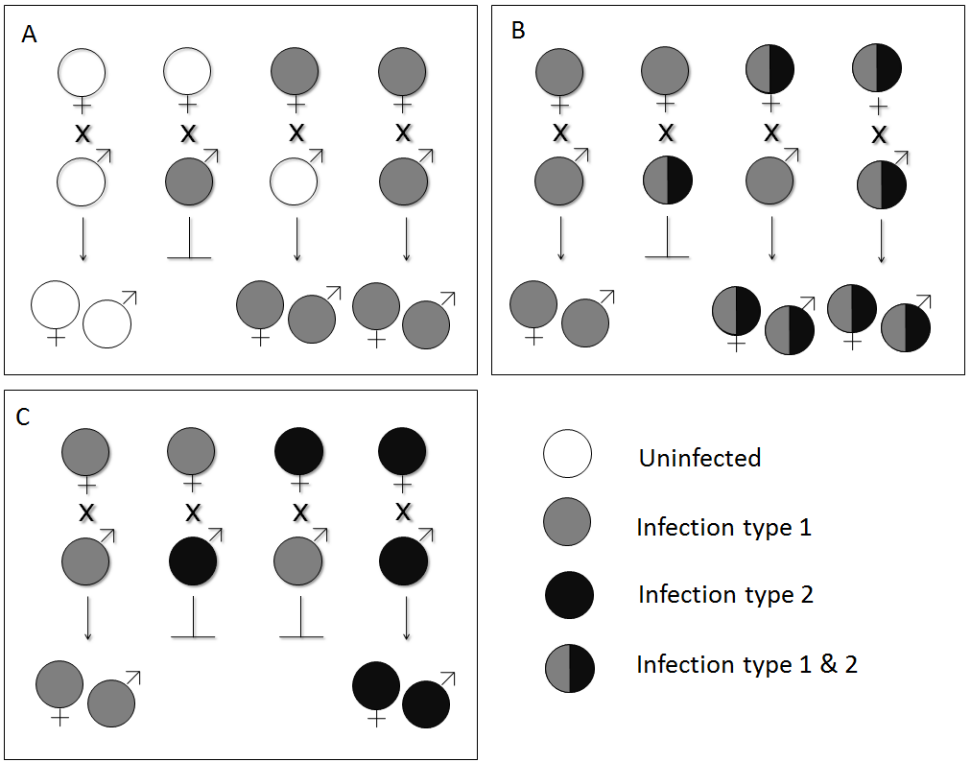


**Figure 1.1.** Examples of cytoplasmic incompatibility. (A) Unidirectional CI is induced between *Wolbachia* infected and uninfected individuals. Infected females are compatible with both infected and uninfected males while uninfected females that mate with infected males produce reduced progeny, resulting in a reproductive advantage conferred on infected individuals. (B) Additive pattern of CI results in unidirectional CI in a population infected with multiple infections. Superinfected (i.e., double infected) females are compatible with both single infected or superinfected males, while single infected females that mate with superinfected males produce reduced progeny, resulting in a reproductive advantage on superinfected individuals. (C) Bidirectional CI is induced between individuals infected with different *Wolbachia* infection types. The strain is in the majority or expresses higher level of CI is expected to replace the population.

## Chapter Two

## Pathogenicity of life-shortening *Wolbachia* in *Aedes albopictus* after transfer from *Drosophila melanogaster*

### Introduction

*Wolbachia* have been identified within a diverse array of invertebrates, where infections are responsible for a variety of host effects including male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI) (O'Neill et al. 1997). The CI phenotype is characterized by early embryonic arrest and a reduction in the number of viable progeny (Callaini et al. 1997, Hoffmann and Turelli 1997, Charlat et al. 2001, Tram et al. 2006). Strict maternal inheritance via embryonic cytoplasm is observed with *Wolbachia*, and while *Wolbachia* numbers can be high in testes (McGraw et al. 2001), transmission of the infection to offspring via males has not been reported (Hoffmann et al. 1998, Xi et al. 2005b). Instead, an unidentified "modification" of sperm acts to initiate CI in fertilized embryos, unless "rescued" by a compatible *Wolbachia* infection in their mates (Charlat et al. 2001). The cost of CI to hosts falls upon uninfected females and infected males within the host population, and since males are a dead-end for *Wolbachia* infection, the resulting dynamics can lead to the spread of infection above an unstable equilibrium threshold (Hoffmann and Turelli 1997).

*Wolbachia* are generally described as "reproductive parasites," and *Wolbachia*/host interactions include examples that span the symbiosis spectrum. Field and laboratory studies support hypothesized trends from pathogenicity toward commensalisms/mutualism (Herre et al. 1999, McGraw et al. 2002, Weeks et al. 2007).

Since mutualistic examples are hypothesized to represent older associations, it follows that maladapted symbioses will be more common among new associations, including artificially generated infections. It is surprising therefore, that additional examples of pathogenic *Wolbachia* symbioses have not been identified to date, especially given examples of *Wolbachia* transinfection. To date, there are two reported examples of pathogenic *Wolbachia*: an artificially-generated association between the isopod *Porcellio dilatatus* and *Wolbachia* injected from *Armadillium* (Bouchon et al. 1998) and the *w*MelPop *Wolbachia* infection in *Drosophila* (Min and Benzer 1997). Both examples are similar in that host mortality occurs relatively late, associated with *Wolbachia* over-proliferation in adult tissues (Juchault et al. 1974, Min and Benzer 1997). A prior artificial transfer of the *w*MelPop infection into *D. simulans* led to a transient exaggeration of pathogenic effects, which were ameliorated in later generations (McGraw et al. 2001, 2002).

A recent report of the stable introduction of *w*MelPop into the medically important mosquito disease vector *Aedes aegypti* (McMeniman et al. 2009) suggests a potential strategy to control disease transmission utilizing the heritable *Wolbachia* infection. Since a female mosquitoes must survive an extrinsic incubation period (EIP) to transmit dengue or other pathogens, a *Wolbachia*-induced shift in the population age structure toward younger females is expected to reduce pathogen transmission (Brownstin et al. 2003, Cook et al. 2008).

*Aedes albopictus* (Asian Tiger Mosquito) is a globally invasive mosquito, which has spread via accidental human transport and competitive dominance, resulting in its displacement of numerous resident mosquito populations (Rai 1991, Gratz 2004, Reiter et

al. 2006). Its relevance as a disease vector has been elevated recently due to its role in recent Chikungunya outbreaks (Enserink 2006, Josseran et al. 2006, Bonilauri et al. 2008, Simon et al. 2008).

Populations of *A. albopictus* are normally superinfected with two *Wolbachia* strains: *w*AlbA and *w*AlbB (Sinkins et al. 1995, Kittayapong et al. 2002b). The infection is among the most mutualistic of associations described for *Wolbachia* in insects (Dobson et al. 2004). Here, we introduced *w*MelPop into *A. albopictus* as the first step toward modifying age structure of an *A. albopictus* population in order to decrease disease transmission such as dengue. However, the *w*MelPop infection in *A. albopictus* was maladaptive and provided an extreme example of *Wolbachia* as a pathogen.

**Materials and Methods**

**Insect strains.** Experiments were conducted using the *w*MelPop infected colony of *Drosophila melanogaster* ($w^{1118}$) (Min and Benzer 1997), naturally superinfected *A. albopictus* (Hou and IH strains) (Sinkins et al. 1995, Dobson et al. 2004) and an aposymbiotic (i.e., uninfected) *A. albopictus* strain (HT1) (Dobson and Rattanadechakul 2001). *Drosophila* and mosquito strains were maintained as described previously (Roberts 1998, Dobson et al. 2001).

**Microinjection.** Injection techniques for embryonic transinfection of mosquito and *Drosophila* were as previously described (Xi et al. 2006). Injection needles were pulled from quartz microcapillaries (#QF 100-70-7.5; Sutter Instrument Co., Novato, CA) using a P2000 (Sutter Instrument Co., Novato, CA). *w*MelPop-infected cytoplasm was withdrawn from the posterior pole of donor $w^{1118}$ embryos and injected into the posterior

pole of HT1 embryos using an IM300 microinjector (Narishige Scientific; Tokyo, Japan). Injected embryos were transferred onto wet filter paper, incubated at 27 ± 2ºC, 75 ± 10% RH for five days and then submerged in deoxygenated water. Resulting larvae ($G_0$) were reared using standard conditions (Dobson et al. 2001) and pupae were isolated as virgins. Eclosing females were mated with HT1 males (i.e. uninfected), blood fed, allowed to oviposit and then PCR assayed to determine their *Wolbachia* infection status. Females failing to produce eggs were not tested.

**PCR amplification and fluorescence *in situ* hybridization.** Two primer sets were used to confirm *w*MelPop infection status: *w*MelPop specific primers (VNTR141F/141R) (Riegler et al. 2005) and general *Wolbachia wsp* primers (81F/691R) (Zhou et al. 1998). DNA was extracted from adult mosquitoes as described previously (Brelsfoard et al. 2008). PCR amplification was performed in 20µl reaction volumes using Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) following the manufacturer's instructions. A MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used to perform 35 cycles of 94°C, 1 min; 55°C, 1 min and 72°C, 1 min. Template quality was confirmed in samples failing to amplify *Wolbachia* DNA using 12S mitochondrial primers as previously described (O'Neill et al. 1992). Fluorescent In Situ Hybridization (FISH) staining was performed according to Xi et al. (Xi et al. 2005b).

**HTM curing.** *Wolbachia* was removed from $G_{10}$ HTM using tetracycline, following the methods described previously (Dobson and Rattanadechakul 2001). The absence of *Wolbachia* in the resulting HTMT line was confirmed by PCR (10 females and 10 males) for two consecutive generations after tetracycline treatment. To minimize the potential

for direct tetracycline effects, crosses with HTMT individuals were performed two generations after tetracycline treatment.

**Longevity, CI and fecundity assays.** For longevity assays, $G_5$ and $G_{13}$ eggs were hatched and the resulting larvae were reared under optimal rearing conditions (i.e., low larval density and liver powder provided *ad libitum*). Newly emerged adults (10 females and 10 males) were placed in cages (n = 20) and provided with a constant 10% sucrose solution. An anesthetized mouse was provided weekly for blood feeding (PHS Assurance #A3336-01). An oviposition cup lined with wet paper was continuously available, with weekly exchanges. Dead mosquitoes were collected at 12-hour intervals and sex determined until all individuals in a cage died. Longevity of females and males were compared separately by Kaplan-Meier logrank test (JMP 7.0.2; SAS Institute; Cary, NC).

For the CI and fecundity assays, $G_{13}$ eggs were used. Rearing of larvae and adults, blood feeding, and egg collection was performed as described above. Eggs from the first batch were hatched after five days of maturation, and the resulting egg number and arcsine transformed hatch rates were compared using ANOVA with post hoc Tukey HSD at $P < 0.05$ (SPSS 11.5; SAS Institute; Cary, NC).

To assess the effects of time (gonotrophic cycle) and parental types used in crosses, a repeated measures ANOVA test were performed (SPSS 11.5; SAS institute; Cary, NC). If the Mauchly's test indicated violation of the sphericity assumption, degrees of freedom were corrected using Huynh-Feldt estimates. Multiple comparisons between cross types used post hoc Tukey HSD.

To confirm insemination, spermathecae were checked in a subset of females by dissecting in Ringer's solution and observing under a dissecting microscope for sperm. Embryonic development was characterized by attaching eggs to double-sided tape (Scotch 665; St. Paul, MN) on a slide glass in a drop of Clorox (Oakland, CA) for 30 min, and observed using an Olympus IX70 fluorescence microscope and photographed using Magnafire software (Optronics; Goleta, CA). The relative proportions of developmental stages for the three groups were compared among cross types using ANOVA with post hoc Tukey HSD at $P < 0.05$.

**Results**

Cytoplasm from *w*MelPop infected *Drosophila* embryos was injected into aposymbiotic *A. albopictus* embryos (HT1 strain), resulting in 13 females ($G_0$) from five microinjection experiments, eight of which were infected and produced progeny allowing establishment of isofemale lines (Table 2.1). The "HTM" isofemale line was selected for additional characterization, based upon the relative stability of maternal inheritance. At $G_{10}$, the HTM line was subdivided and one of the resulting lines was closed (i.e., females no longer out-crossed with HT1 males, but mated with HTM males). Out-crossing with HT1 males continued in the other line.

In the out-crossed HTM line, high maternal inheritance continued through $G_{10}$, with 99.8 ± 0.13% (mean ± standard error [SE]; n = 150) and 100% (n = 38) infection detected in females and males, respectively. In contrast, the infection frequency dropped in the closed line, resulting in the loss of *Wolbachia* infection within three generations in the absence of selection for *w*MelPop infection (Fig. 2.1A). In a second experiment,

closing of the HTM line was associated with a similar decline in infection frequency at $G_{11}$ without selection for *w*MelPop infection (Fig. 2.1A). The line was split at $G_{11}$ and females were either mated by HTM or HT1 males and selected for *w*MelPop infection. Although both closed and out-crossed lines were selected for *w*MelPop infection, only the out-crossed line subsequently restored infection up to 91% at $G_{13}$ (Fig. 2.1A), and the closed line lost the infection (Fig. 2.1A). As an additional test for paternal effects on *Wolbachia* infection frequency, $G_{13}$ HTM females were mated with either HT1 or HTM males. PCR assays of the resulting progeny demonstrated 91.3 ± 2.7% and 57.0 ± 2.4% (mean ± SE) infection frequencies, respectively (Fig. 2.1B). The absence of paternal transmission of the *w*MelPop infection to progeny was confirmed by PCR assays of 60 first instar larvae resulting from three gonotrophic cycles of HT1 females crossed with HTM males, which resulted in no infection in progenies.

Egg hatch rates resulting from the four cross combinations between HTM and HT1 individuals were examined (Fig. 2.2). Significantly reduced hatch rates (multiple comparisons with post hoc Tukey HSD, $P < 0.0001$) were observed in all three cross types that included HTM individuals (female or male) relative to crosses between uninfected individuals. No difference in egg hatch was observed between crosses that included HTM individuals. The egg hatch rates remained consistent across multiple gonotrophic cycles (repeated measures ANOVA, Huynh-Feldt correction; $F = 2.6$, df = 3.16, $P = 0.060$). To examine the possibility that reduced egg hatch resulted from failure of HTM males to inseminate females, spermathecae were examined from females in each cross type (n = 25), and sperm were observed in 100% of the spermathecae. While female fecundity was observed to decrease significantly across gonotrophic cycles in all

four cross types (repeated measures ANOVA, Huynh-Feldt correction; $F = 124.1$, df = 4.58, $P < 0.0001$), a comparison of fecundity between cross types revealed a difference between the HTM × HTM (female x male) and HT1 × HT1 cross only ($P = 0.045$) (Fig. 2. 2). Removal of the *w*MelPop infection from the HTM line via tetracycline treatment had no effect on fecundity. The fecundity of the cured HTMT line did not differ significantly from HT1 crosses. In contrast, removal of the *Wolbachia* infection in the HTMT line restored egg hatch to levels indistinguishable from the compatible HT1 crosses (Table 2.2).

While egg hatch failure can result from multiple reasons, the typical CI phenotype is characterized by early embryonic arrest (Tram et al. 2006). Embryonic bleaching was used to examine the development of eggs failing to hatch from HTM crosses. Unhatched eggs were assigned to one of three categories: no development, intermediate development, and visible eye spot (Fig. 2.3). A majority of unhatching embryos from HT1 × HT1 crosses showed late-stage development (i.e., eye spots). In contrast, the HT1 × HTM cross predominantly resulted in no development among hatching eggs. Both cross types that included HTM females were similar, resulting in proportionally more eggs displaying intermediate levels of development. For the HTM larvae from the out-crossed line that successfully hatch, high survivorship was observed (i.e., similar to naturally-infected mosquitoes) (Islam and Dobson 2006): 258 pupae/284 larvae (91%) survived to pupate and 94% of the resulting pupae emerged as adults (49% females).

Fluorescent In Situ Hybridization (FISH) was used to visualize *Wolbachia* in the HTM, HT1, HTMT and IH (i.e., naturally-infected) embryos (Fig. 2.4). HT1 and HTMT were similar in that no *Wolbachia* were observed. Naturally-infected embryos displayed

a pattern of *Wolbachia* infection focused in the periphery and poles, similar to prior descriptions (Xi et al. 2005b). In contrast, the *w*MelPop infection was higher in HTM embryos and distributed throughout the embryo.

An initial comparison of HTM and HT1 longevity was conducted at $G_5$, showing reduced longevity of HTM females relative to HT1 females (Kaplan-Meier logrank; $\chi 2 = 4.622$, df = 1, $P = 0.032$) (Fig. 2.5). The median longevities of HTM and HT1 females were 41 and 54 days, respectively. No difference was observed between HTM and HT1 males, with median ages of 30 and 25 days, respectively ($\chi^2 = 3.286$, df = 1, $P = 0.070$). Repeating the longevity assay at $G_{13}$ (Fig. 2.5), HTM females again were observed to be significantly shorter-lived (median age of 52.5 days) relative to HT1 females (median age of 57.5 days) ($\chi^2 = 5.298$, df = 1, $P = 0.0213$). Similar to the $G_5$ longevity assay, no difference was observed between HTM and HT1 males, with median ages of 26 and 24.5 days, respectively ($\chi^2 = 3.0377$, df = 1, $P = 0.0814$).

**Discussion**

The results presented here demonstrate that, although *A. albopictus* is permissive to the *w*MelPop *Wolbachia* type, the resulting infection can be best categorized as a pathogenic symbiosis. The results of microinjection experiments are similar to prior transinfection experiments (McGraw et al. 2002, Reynolds et al. 2003), showing that *A. albopictus* susceptibility to *w*MelPop is not atypical. However, the resulting phenotype of the HTM strain is unlike other *Wolbachia* infections and the most pathogenic *w*MelPop association reported to date. The reduced fitness is due primarily to low egg hatch of HTM females, which results regardless of male infection status. Maladaptation of *w*MelPop was pronounced in *A. albopictus* compared to the results of establishing

*w*MelPop in naturally uninfected *A. aegypti* (McMeniman et al. 2009). Examination of unhatched HTM embryos reveals a high proportion at an intermediate level of development. Late-arrested embryos have been described for CI induction in *Cx. pipiens* when both females and males are *Wolbachia* infected, suggesting that an infection in females may facilitate limited morphogenesis (Duron and Weill 2006). There are reports also of "suicide" *Wolbachia* infections, capable of modifying but not rescuing (Zabalou et al. 2008). While partial rescue can potentially explain events resulting in crosses between HTM individuals, it does not explain the low egg hatch resulting from crosses of HTM females and uninfected males, which in theory would not induce CI. Removal of the *w*MelPop infection (i.e., antibiotic clearing of the HTMT strain) restored normal egg hatch, demonstrating that the low egg hatch phenotype is not explained by the HTM genotype or mitotype which possibly has been changed during microinjection of *w*MelPop.

Examination of HTM oocytes shows an unusually high density of *Wolbachia*, suggesting that *Wolbachia* over-replication in oocytes is responsible for the low egg hatch resulting from HTM females. This is similar to a reported transfer of *w*MelPop to *D. simulans* in which the *w*MelPop infection was associated with a drop in egg hatch (McGraw et al. 2002). In the prior experiment however, the egg hatch reduction was relatively small in *D. simulans* and was transient, returning to normal egg hatch levels after five generations of transinfection experiments. Similar to results in transinfected *D. simulans* (McGraw et al. 2002), lower fecundity was observed in HTM females consistent with higher costs associated with *w*MelPop infection.

*Wolbachia* has been shown to change phenotype upon transfer between hosts (Boyle et al. 1993, Sasaki et al. 2002, Zabalou et al. 2008). With the *w*MelPop infection in *Drosophila*, a range of CI penetrance has been described (Min and Benzer 1997, McGraw et al. 2002, Reynolds et al. 2003). Although CI induction by *w*MelPop in *A. albopictus* was not initially apparent, based upon the pattern of egg hatch, subsequent examination of embryonic development reveals a cross pattern that is consistent with CI. Specifically, the cross between uninfected females and HTM males is different from the remaining cross types, resulting in significantly more embryos that are arrested in early development, which is diagnostic of CI. However, the CI is incomplete (~20% hatch) and relatively weak compared to that resulting in crosses of the natural superinfection (< 1% hatch) and CI resulting from previously generated *A. albopictus* transinfected lines (< 15% hatch) (Xi et al. 2005b, Xi et al. 2006). Importantly, the *w*MelPop infection in HTM can rescue the CI modification (i.e., mod+ resc+) (Charlat et al. 2001), resulting in broods indistinguishable from the HTM × HTM and HTM × HT1 crosses.

Similar to the phenotype in the original *D. melanogaster* host, the *w*MelPop infection is associated with reduced adult longevity. HTM longevity assays were conducted at $G_5$ and then repeated at $G_{13}$. The latter crosses were conducted because prior studies showed an attenuation of the *w*MelPop phenotype with time (McGraw et al. 2002). The later assay is expected also to ameliorate potential inbreeding effects resulting from transinfection and isoline selection methods. In both the $G_5$ and $G_{13}$ assays, HTM female adults were observed to be significantly shorter lived. However, the reduction of female longevity was not as severe as that observed in *D. melanogaster, D. simulans,* or *A. aegypti* (Min and Benzer 1997, McGraw et al. 2002, McMeniman et al.

2009), where the lifespan was approximately halved by *w*MelPop infection. No effect of *w*MelPop on adult male longevity was observed.

High maternal transmission of *w*MelPop is observed in HTM, when out-crossed to uninfected males. However, lower infection frequency was observed resulting from crosses of HTM females and HTM males. This apparent paternal effect on the *Wolbachia* infection frequency among progeny is unexpected, and additional study is required to determine whether this represents a direct effect on embryonic infection frequency and/or infection level among progeny or whether this results from an indirect downstream effect (e.g., differential larval competition/survivorship favoring rare uninfected individuals). The absence of paternal transmission was confirmed, which is consistent with prior reports describing that paternal transmission of *Wolbachia* as rare or absent (Hoffmann and Turelli 1988, McGraw et al. 2001).

While prior works suggest that, with time, the HTM strain may evolve toward a mutualistic association (McGraw et al. 2002, McMeniman et al. 2009), the current symbiosis is maladaptive. With weak CI and high fitness cost, the *w*MelPop infection would be unlikely to spread into an uninfected *A. albopictus* population. The opportunity for expansion is further reduced by the low maternal transmission that results in crosses between HTM individuals. The results, especially in comparison with related work (McMeniman et al. 2009), demonstrate the unpredictability of phenotypes resulting in artificial *Wolbachia*/host associations, which is an important consideration in extending an age structure modification strategy to additional systems (Sinkins and Gould 2006, Bourtzis 2008).

**Table 2.1.** Survival and infection status of *Aedes albopictus* microinjected with *w*MelPop *Wolbachia*.

| Experiment | Percent hatch (Larvae/ injected Egg) | Percent pupation (Pupae/ larvae) | Percent eclosion (Adults/ pupae) | $G_0$ infection status (percent infected) | |
|---|---|---|---|---|---|
| | | | | Female (infected female/ total tested) | Male (infected male/ total tested) |
| 1 | 17.5% (17/97) | 94.1% (16/17) | 100.0% (16/16) | 66.7% (4/6) | 75.0% (6/8) |
| 2 | 5.2% (7/134) | 100.0% (7/7) | 71.4% (5/7) | NA (0/0) | 50.0% (1/2) |
| 3 | 15.7% (21/134) | 90.5% (19/21) | 100.0% (19/19) | 60.0% (3/5) | 55.6% (5/9) |
| 4 | 4.8% (6/126) | 83.3% (5/6) | 60.0% (3/5) | NA (0/0) | 100.0% (2/2) |
| 5 | 5.5% (6/110) | 100.0% (6/6) | 100.0% (6/6) | 50.0% (1/2) | 100.0% (3/3) |

**Table 2.2.** Fecundity (number of eggs ± SE) and hatch rate (percent egg hatch ± SE) resulting from crosses of the HTMT strain. Differing superscripted letters indicate significant difference ($P < 0.05$; ANOVA).

| Female | Male | Egg number | Percent hatch |
|---|---|---|---|
| HTMT | HT1 | 840 ± 143; n = 5 $^{a}$ | 88.8 ± 1.4; n = 5 $^{b}$ |
| HT1 | HTMT | 597 ± 78; n = 5 $^{a}$ | 65.3 ± 9.6; n = 5 $^{c}$ |
| HTMT | HTMT | 646 ± 77; n = 5 $^{a}$ | 80.0 ± 2.5; n = 5 $^{bc}$ |
| HT1 | HT1 | 687 ± 54; n = 5 $^{a}$ | 83.1 ± 2.7; n = 5 $^{bc}$ |
| HTM | HTM | 541 ± 100; n = 3 $^{a}$ | 30.4 ± 3.2; n = 3 $^{d}$ |

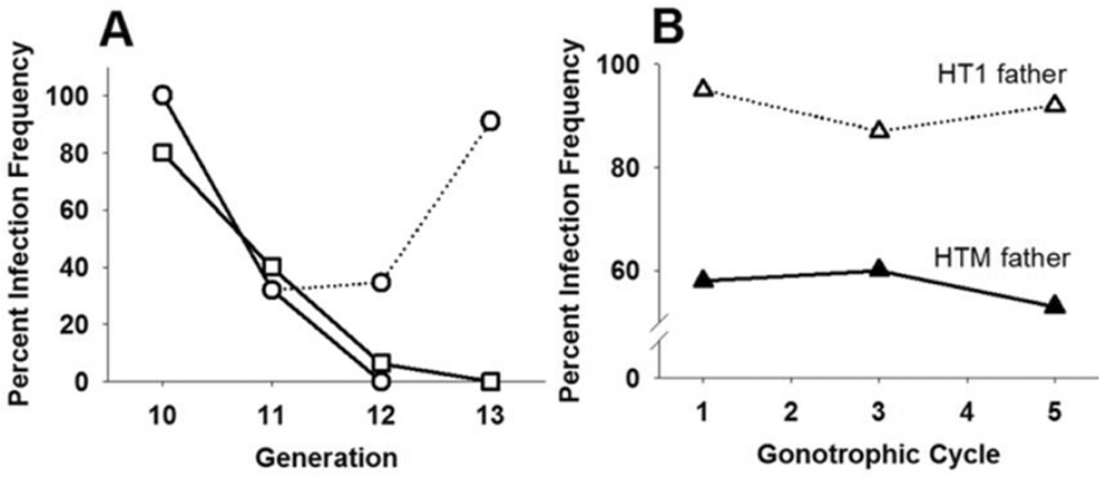


**Figure 2.1.** Infection frequency among progeny of HTM females mated with either HTM males (solid line) or HT1 males (broken line). (A) Experiment I (□), closed HTM line with no selection; Experiment II (○), closed and out-crossed line selecting for infection at $G_{11}$. (B) Experiment III, closed and out-crossed line at $G_{13}$ across five gonotrophic cycles. Closing the population resulted in loss of infection in experiments, whereas outcrossing restored infection.

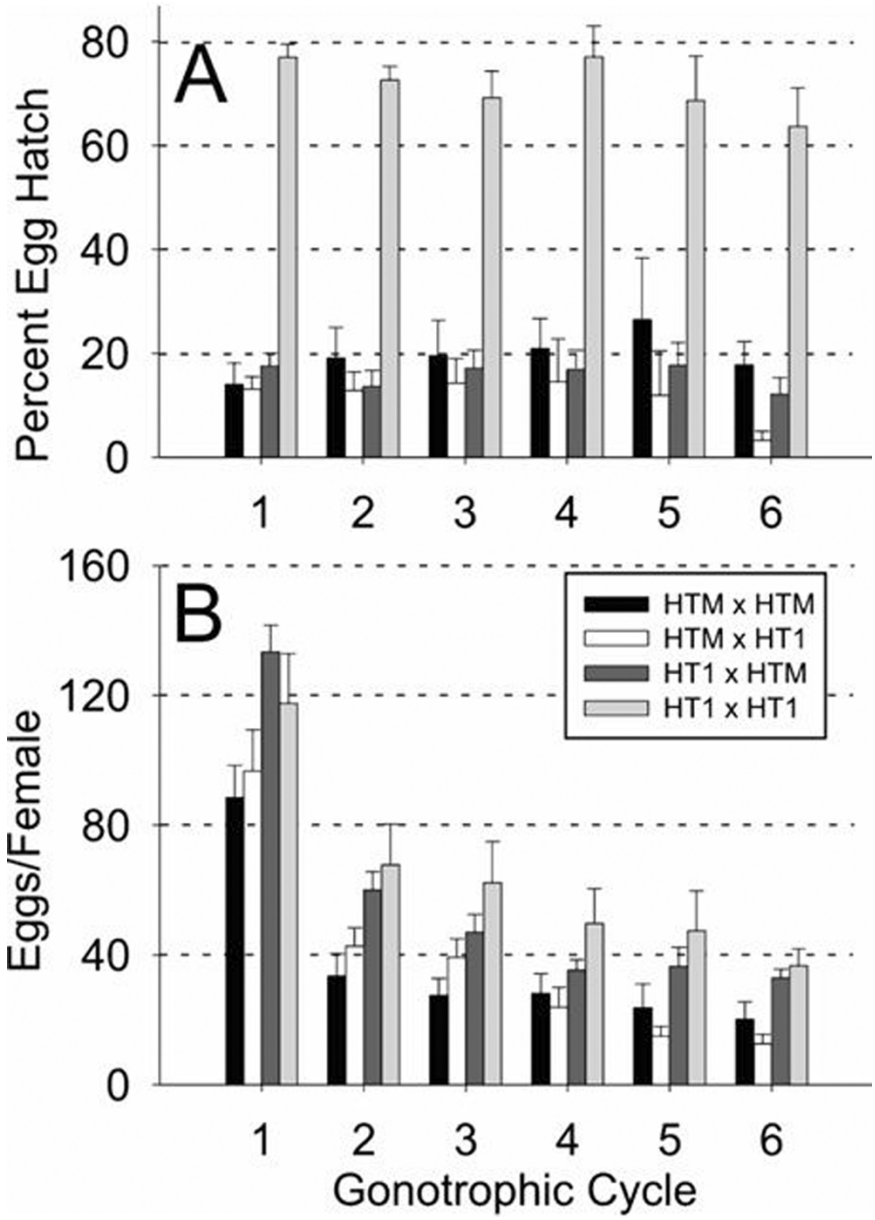


**Figure 2.2.** (A) Percent egg hatch and (B) egg number resulting from crosses between HTM and HT1 *A. albopictus* strains infected with *w*MelPop *Wolbachia* and uninfected, respectively. Data are shown for six gonotrophic cycles. Crosses are shown as female × male. Error bars = SE. Unlike the typical CI phenotype, *w*MelPop resulted in low hatch rates from cross types expected to be compatible (i.e., HTM × HTM and HTM × HT1).

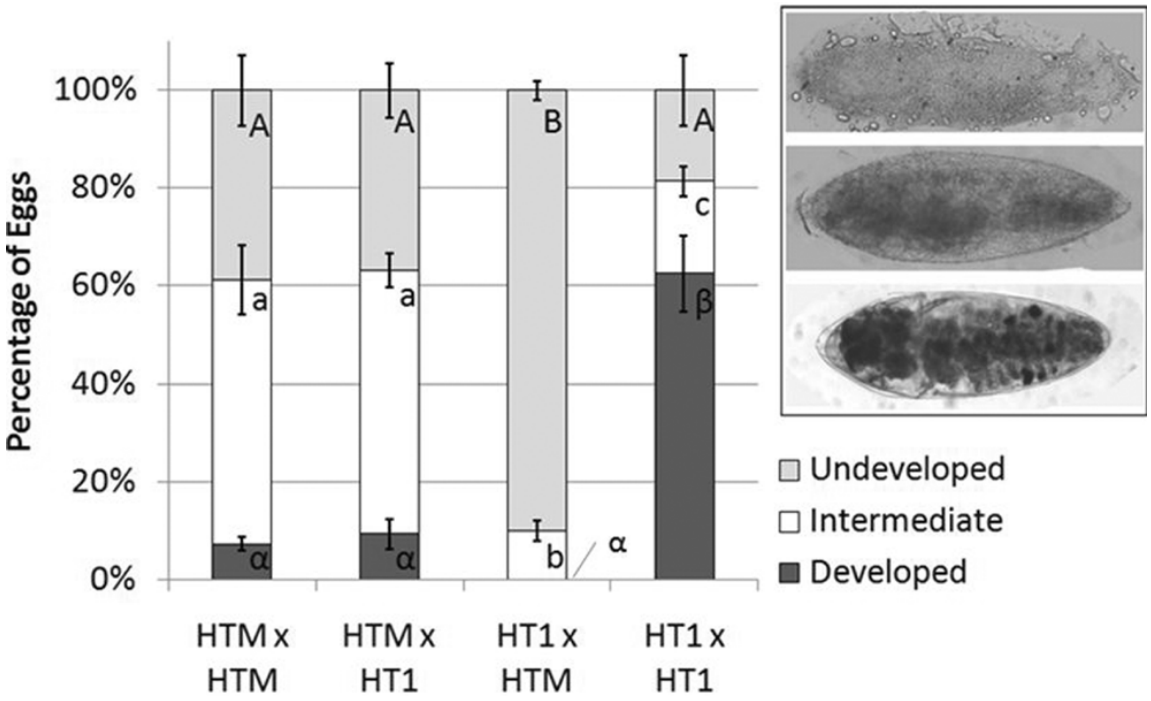


**Figure 2.3.** Characterization of embryonic development in unhatched embryos resulting from the four possible cross types between the HTM and HT1 strains of *A. albopictus*, which are infected with *w*MelPop *Wolbachia* and uninfected, respectively. Bleached embryos were assigned to three developmental categories: Undeveloped, Intermediate and Developed (inset, top to bottom). Differing letters correspond to significant differences within each embryonic category ($P < 0.05$). Crosses are shown as female × male. Error bars = SE.

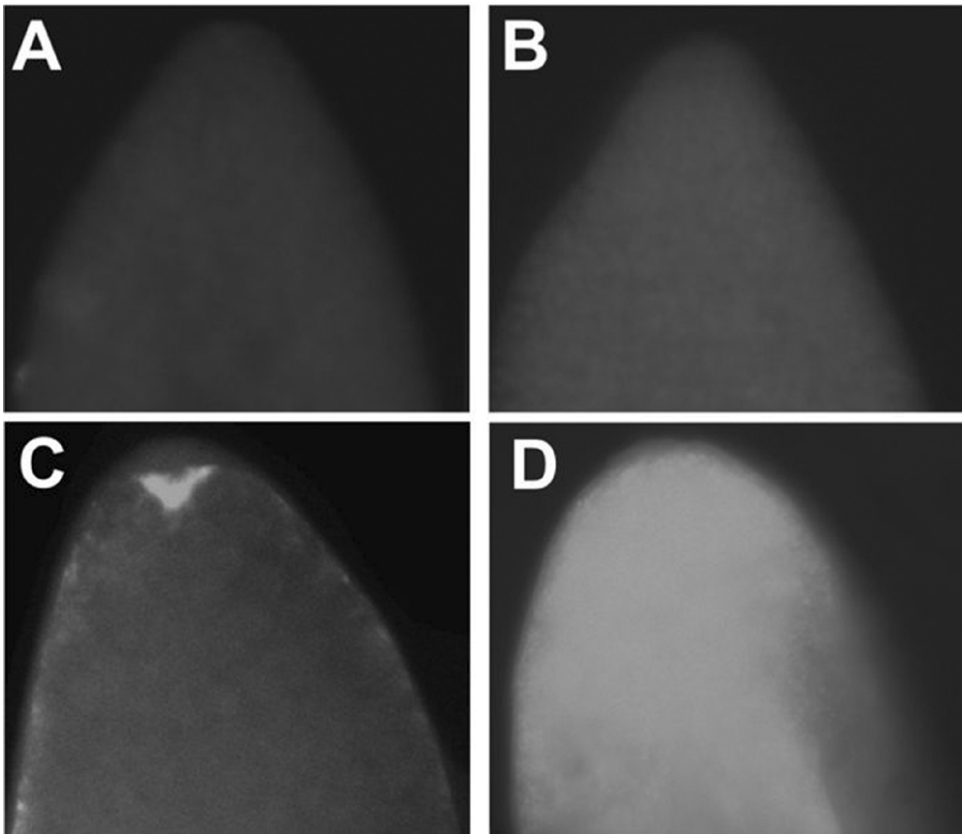


**Figure 2.4.** Fluorescent In Situ Hybridization used to visualize *Wolbachia* distribution in *A. albopictus* oocytes from the strains: (A) HT1, aposymbiotic; (B) HTMT, tetracycline-cured HTM; (C) IH, naturally superinfected strain; (D) HTM, *w*MelPop-transinfected. A high density of *Wolbachia* is observed in HTM embryos infected with *w*MelPop, and the infection is more broadly distributed relative to that observed in naturally-infected embryos.

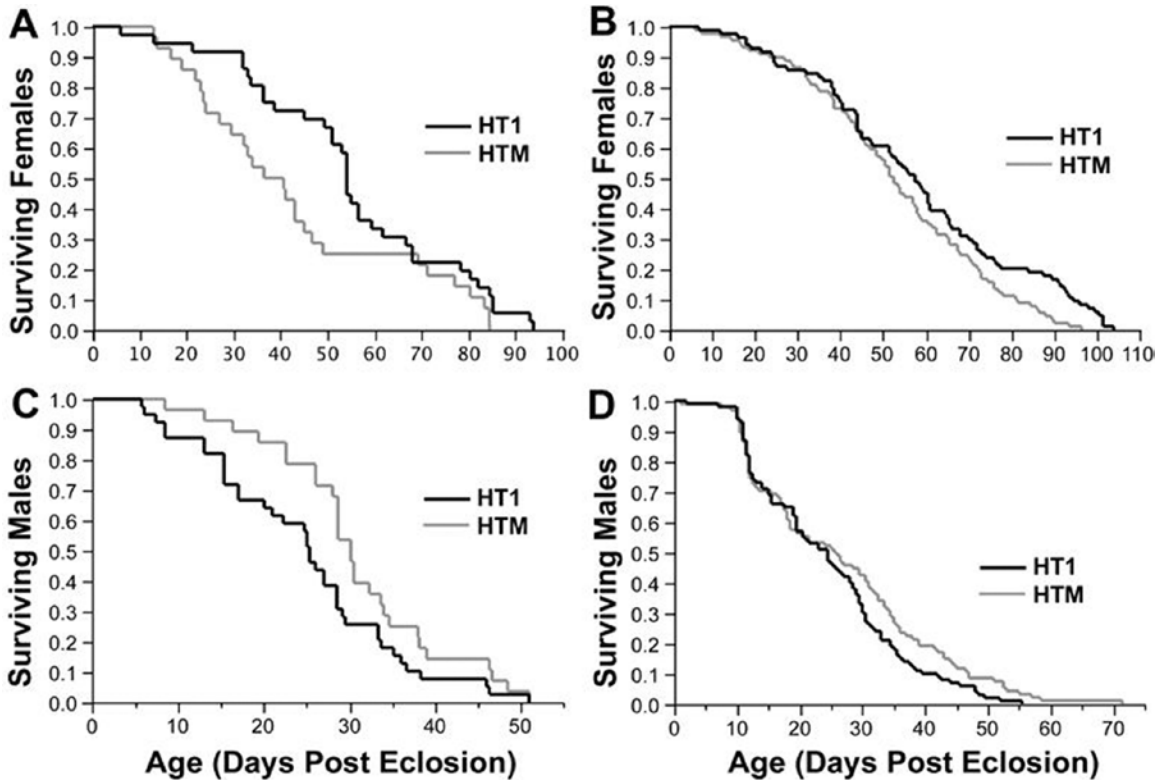


**Figure 2.5.** Longevity of *A. albopictus* (A, B) females and (C, D) males that are either infected with the *w*MelPop (HTM strain) or are uninfected (HT1 strain) at generation five (A, C) and thirteen (B, D). HTM infection resulted in reduced lifespan for infected females but not for males.

## Chapter Three

## Characterization of triple *Wolbachia* infection in *Aedes albopictus* after introduction of *w*MelPop from *Drosophila melanogaster*

### Introduction

*Wolbachia* are maternally inherited, intracellular bacteria, that infect a variety of arthropods and nematodes (O'Neill et al. 1997). *Wolbachia* are responsible for diverse impacts on a host reproduction including male killing, parthenogenesis, feminization and cytoplasmic incompatibility (CI). The modifications can be beneficial to *Wolbachia*, providing infected hosts with a reproductive advantage that can promote the spread of *Wolbachia*. In mosquitoes, *Wolbachia* induces CI, which causes a decrease or absence of brood hatch when infected males mate with females that are uninfected or infected with a different *Wolbachia* type (Charlat et al. 2001, Werren et al. 2008). Individuals can be infected with two or more *Wolbachia* strains (i.e., superinfections), which results in diverse CI patterns (Fig. 3.1).

There has been considerable interest in using *Wolbachia* based strategies to control insect vectors of disease and disease transmission. Strategies include: (a) *Wolbachia* based Incompatible Insect Technique (IIT) strategies, analogous to the sterile insect technique and (b) population replacement strategies that focus on replacing natural populations with individuals expressing a desired phenotype (O'Neill et al. 1997, Werren 1997). As an example of the latter, natural *Wolbachia* infections (*w*MelPop) have been associated with a life-shortening phenotype, suggesting a strategy to control disease transmission (Min and Benzer 1997, McMeniman and O'Neill 2010). In order to

complete the disease transmission cycle, female mosquitoes must survive the extrinsic incubation period (EIP). Young females can reproduce, enabling the spread of *w*MelPop infection via CI yet these females do not survive long enough for the parasite to complete the extrinsic incubation period. Thus, skewing the age structure toward a younger population may decrease disease transmission. Furthermore, *w*MelPop has also been identified to interfere with the infection of human pathogens within the mosquitoes (Moreira et al. 2009b), providing the population possessing the *w*MelPop infection with potential resistance to disease.

Mosquitoes are capable of utilizing multiple sources of blood, which can affect fecundity and egg hatch rate in some mosquito species (Woke 1937, Bennett 1970, Gubler 1970, Mather and DeFoliart 1983, Xue et al. 2009). *Wolbachia* have also been shown to affect fecundity and egg hatch (Vavre et al. 1999, Dobson et al. 2002b, Fry et al. 2004, McMeniman et al. 2011), thus it is important to examine for an interaction between blood meal type and *Wolbachia* infection effects on fecundity and hatch.

*Aedes albopictus* (Asian Tiger Mosquito) is an opportunistic generalist feeder (Savage et al. 1993, Niebylski et al. 1994, Delatte et al. 2010), utilizing blood from multiple host species including human and non-human animals. It is also an efficient vector of multiple arboviruses and filaria species (Francy et al. 1990, Rai 1991, Moore and Mitchell 1997, Cancrini et al. 2003, Gratz 2004). Recently, *A. albopictus* has implicated as a vector of the Chikungunya virus, highlighting its medical importance as vector species (Enserink 2006, Josseran et al. 2006, Bonilauri et al. 2008, Simon et al. 2008). *A. albopictus* is also naturally infected with *Wolbachia*. Surveys of natural *A. albopictus* populations suggest that they are consistently infected with two *Wolbachia*

types, *w*AlbA and *w*AlbB, throughout its geographical distribution (Sinkins et al. 1995, Zhou et al. 1998, Armbruster et al. 2003).

Here, the *w*MelPop infection is introduced into a strain of *A. albopictus* naturally superinfected with *Wolbachia*. Experiments were performed to examine the maternal inheritance rates, CI, and evidence for the life shortening phenotype in *w*MelPop infected mosquito lines. Furthermore, effects of different blood sources on maternal inheritance, fecundity, and egg hatch of the transinfected strains were examined. The results are discussed in relation to the use of *w*MelPop infected *A. albopictus* to modify the age structure of natural populations to inhibit disease transmission and effects of different blood sources.

**Materials and Methods**

**Insect strains.** Microinjection experiments used *w*MelPop infected colony of *Drosophila melanogaster* ($w^{1118}$) , wild type *Aedes albopictus* (IH) and an aposymbiotic *A. albopictus* strain (*UjuTet*, UT) that had been artificially generated by tetracycline treatment to remove the *Wolbachia* infection (Xi et al. 2005a). Rearing conditions were as previously described (Dobson et al. 2001).

**Microinjection.** Injection techniques for embryonic transinfection of mosquito and *Drosophila* were as previously described (Xi et al. 2006). Injection needles were pulled from quartz microcapillaries (QF 100-70-7.5; Sutter Instrument Co., Novato, CA) by using a P2000 (Sutter Instrument Co., Novato, CA). *w*MelPop-infected cytoplasm was withdrawn from the posterior pole of donor $w^{1118}$embryos and injected into the posterior pole of IH embryos by using an IM300 microinjector (Narishige Scientific, Tokyo,

Japan). Injected embryos were transferred onto wet filter paper, incubated at 27 ± 2°C and 75 ± 10% relative humidity for five days, and then submerged in deoxygenated water. Resulting larvae ($G_0$) were reared using standard conditions (Dobson et al. 2001), and pupae were isolated to produce virgins females. Eclosing females were mated with UT (i.e., uninfected) males, blood fed, allowed to oviposit, and then PCR assayed to determine their *Wolbachia* infection status. Females failing to produce eggs were not tested.

**Multiplex PCR amplification.** DNA was extracted from adult mosquitoes as described previously (Brelsfoard et al., 2008). Three primer sets were used to detect triple *Wolbachia* infections; *w*MelPop (IS5F/IS5R; McMeniman et al., 2008), *w*AlbA (183F/691R) and *w*AlbB (328F/691R) (Zhou et al., 1998). PCR amplification was performed in a 12.5µl reaction volume, using a Qiagen multiplex PCR kit, following manufacturer's instructions; 1µl DNA template; 4µl H2O; 1.25µl 10X primer mix; 6.25µl Master Mix) (Qiagen, Valencia, CA). An MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used to perform 35 cycles of 94°C for 30 sec, 57°C for 90 sec, and 72°C for 60 sec. Template quality was confirmed in samples failing to amplify *Wolbachia* DNA by using 12S mitochondrial primers as previously described (O'Neill et al., 1992).

**Fecundity, CI, adult longevity and egg viability.** For the fecundity and CI assays, larvae were reared under optimal conditions (i.e., low larval density and liver powder provided *ad libitum*). Newly emerged adults (10 females and 10 males) were placed in cages with five replicates per treatment and provided with a constant supply of 10% sucrose solution. Human blood using a Hemotek membrane feeder (Discovery

Workshops, Accrington, UK) or an anesthetized mouse (A3336-01; PHS Assurance) was provided for blood feeding. An oviposition cup lined with wet paper (Anchor Paper Company, St. Paul, MN) was continuously available, with weekly exchanges. Eggs from the first batch were hatched after five days of maturation, and the resulting egg number and hatch rates were compared using one-way analysis of variance (ANOVA) with post hoc Tukey honestly significant difference (HSD) at $P = 0.05$. To assess the potential effect of blood source on fecundity or egg hatch rate of YFU line, student *t*-test or two-way ANVOA test was used (JMP 8.0.1; SAS Institute, Cary, NC).

For adult longevity assays, rearing of larvae and adults, blood feeding, and egg collection were performed as described above. Dead mosquitoes were collected twice per day until all individuals in a cage died. The survivorship of females and males was compared separately by using a Kaplan-Meier log-rank test (SPSS 17.0; SAS Institute, Cary, NC).

YFU eggs were collected by providing human blood via Hemotek artificial blood feeding tools, and the egg paper was subdivided into five groups with 43 ± 1.9 eggs (mean ± standard error [SE]; n = 95). Eggs were matured by wetting 3 days at 100% relative humidity and dried for two days. Eggs were stored at 28 ± 2°C and 75 ± 10% RH and hatched 5, 8, 16, 30 and 51 days post oviposition for three days by adding liver-powder solution *ad libitum*. Statistical analysis tested effect of time on egg hatch rate using multiple linear regression model and egg hatches were compared between cross types (JMP 8.0.1; SAS Institute, Cary, NC).

## Results

**Embryonic microinjection and maternal inheritance**. Cytoplasm from *w*MelPop-infected *Drosophila* embryos was microinjected into naturally super-infected *A. albopictus* (IH strain) embryos (Cite methodology). The resulting infected females ($G_0$) were used to establish isofemale lines (Table 3.1). Three isofemale lines were selected for stable infection during the first six generations while out-crossing with uninfected *A. albopictus* (UT). The resulting strain was designated as YFU. The YFU line was subdivided at $G_{17}$; one line was closed (i.e., mated with YFU males) and the other line continued out-crossing with UT. At $G_{20}$, the out-crossed line was subdivided further, resulting in four lines, conducting selection for *Wolbachia* infection at every generation.

When fed on human blood, the maternal inheritance rates (MIR) was not observed to be affected by the paternal infection type. In the out-crossed line, MIR of triple *Wolbachia* infection during $G_{7\sim29}$ was 93.96 ± 2.28% (n = 20) (mean ± standard error). Loss of *w*AlbA infection was occasionally detected (2.63 ± 1.92%; n = 20) while loss of *w*AlbB infection was not observed in the out-crossed line. MIR of the closed line was 98.41 ± 1.59% (n = 7) during $G_{19\sim29}$ and it was not significantly different from the out-crossed line (generalized linear model with binomial distribution and Logit link; $\chi^2 = 5.53$, df = 3, $P = 0.13$; JMP 8.0.1; SAS Institute, Cary, NC).

In contrast, an association between MIR and paternal infection type was observed. Specifically, when fed on human blood, YFU females out-crossed with UT males showed a recovery from 75% infection frequency of *w*MelPop at the start of the experiment, to 100% infection after four generations (Fig. 3.2A). A continuing decrease in infection, resulting in the complete loss of infection, was observed in crosses between YFU females

and males, when fed on mouse blood (Fig. 3.2B). Loss of either *w*AlbB or *w*AlbB infection was not detected during this experiment.

In order to exclude potential impact of paternally inherited *w*MelPop on progeny, PCR assays were conducted on six pools of 20 1$^{st}$ instar (L1) resulting from three gonotrophic cycles of IH females crossed with YFU males in the mating experiments using mouse blood. No *Wolbachia* infected progeny were observed (Fig .3.3).

**Fecundity and egg hatch.** Fecundity of YFU and IH was dependent on blood source. When fed with human blood, the egg number did not differ significantly among four cross types (one-way ANOVA; $F_{3,15} = 1.66$, $P = 0.22$) (Fig. 3.4A). However, when fed with mouse blood, fecundity of IH females was higher than YFU females ($F_{3,16} = 10.91$, $P < 0.001$). The interaction effect between blood and infection type of females on fecundity was significant ($F_{3,35} = 22.35$, $P < 0.0001$).

Egg hatch rates resulting from matings between YFU and IH were significantly different when testing for an effect of whether they fed on human or mouse blood. With human blood, egg hatch was the lowest when IH females were mated with YFU males ($F_{3,15} = 14.94$, $P < 0.0001$) (Fig. 3.4.B). In contrast, when fed mouse blood, egg hatch was low from YFU females, regardless of paternal type ($F_{3,16} = 62.02$, $P < 0.0001$). The interaction effect between blood and infection type of females on egg hatch ($F_{3,35} = 13.35$, $P < 0.0001$) was also significant.

Four isofemale lines lost the *w*MelPop infection during selection experiment, and their progeny were pooled to generate genetically similar line (YFUC). Egg hatch was monitored during six generations of YFUC line, which showed similar egg hatch rates (72.37 ± 8.73%; mean ± SD; n = 6) to those observed in crosses between IH individuals

(see Fig. 3.4B). Corresponding generations of the YFU line out-crossed with UT males ($G_{22}$~$G_{27}$) had egg hatch rates of 56.75 ± 15.15% (mean ± SD; n = 6), which showed no significant difference from the egg hatches of YFUC lines ($F_{1,10} = 3.80$, $P = 0.08$).

**Longevity.** Adult longevity was significantly reduced in YFU females compared to IH (Kaplan-Meier log rank comparisons; $\chi^2 = 4.84$, $P = 0.028$) and UT ($\chi^2 = 12.44$, $P < 0.0001$) when fed using mouse blood (Fig. 3.5). The adult longevity of IH and UT females were not significantly different from each other ($\chi^2 = 2.56$, $P = 0.11$). Mean female longevity of YFU, IH and UT were 36 ± 2.3 (mean ± SE), 43 ± 2.6, and 53 ± 3.1 days. The longevity of YFU males was also reduced compared to IH ($\chi^2 = 0.001$, $P < 0.05$) but not different from UT ($X^2 = 0.001$, $P = 0.976$). Longevity was not different between IH and UT males ($\chi^2 = 2.72$, $P = 0.099$).

**Egg viability.** Viability of *A. aegypti* eggs was constant over time for all cross types during 51 days after eggs were generated by feeding human blood; YFU × YFU (Female × Male; $R^2 = 0.0027$, $F_{1, 23} = 0.062$, $P = 0.81$), YFU × IH ($R^2 = 0.0015$, $F_{1, 23} = 0.035$, $P = 0.85$), IH × YFU ($R^2 = 0.023$, $F_{1, 23} = 0.55$, $P = 0.47$), IH × IH ($R^2 = 0.021$, $F_{1, 18} = 0.39$, $P = 0.54$) (Fig. 3.6). Mean egg hatches of four cross types were compared resulting in compatible cross (IH × IH) highest, incompatible cross (IH × YFU) lowest and crosses involved with YFU females (YFU × YFU and YFU × IH) were intermediate (ANOVA with Tukey post hoc; $F_{3, 16} = 236.19$, $P < 0.0001$). Adults emerged from hatching larvae sampled from day five and 51 were all triply infected (n = 20).

**Discussion**

Here, an unusual interaction of *Wolbachia* infection level and blood source was observed, which had significant impacts on host fitness. An infection cost was observed in the fecundity of YFU females only when mouse blood was provided, which was consistent with the previous study (Suh et al. 2009), while no difference was observed with human blood. The level of CI was not significantly different using both blood types. Low egg hatch with mouse blood in the YFU females was consistent with the previous report (Suh et al. 2009), which was even lower than the incompatible cross (i.e., IH females mated with YFU males). Thus, in this case the triple infection is also defined as pathogenic *Wolbachia* infection, as previously described (Suh et al. 2009). In contrast, the egg hatch was ten times higher when YFU females fed on human blood, regardless of male type. The egg hatch from the compatible crosses in YFU females was higher than the incompatible cross, which may endow reproductive advantage to triple infected individuals over the double infected in a panmictic population.

The drastic change in embryo viability of YFU strain was a function of *w*MelPop infection interacting with blood source. The blood effect was consistent in the *A. aegypti* infected with *w*MelPop-CLA (cell-line-adapted), which showed more pronounced difference in egg hatch between human blood with up to 80% and mouse blood with almost 0% egg hatch while no significant effect on egg hatch in aposymbiotic *A. aegypti* (McMeniman et al. 2011). Furthermore, the *Wolbachia* infected *A. aegypti* showed differential response in egg hatch and fecundity depending on blood sources, which suggest the *Wolbachia* infection was associated with utilization of blood source in embryo development (McMeniman et al. 2011).

In recent studies, *Wolbachia* has been shown to be associated with iron homeostasis in the host cell as well as *Wolbachia* utilizing iron for its own survival (Brownlie et al. 2009, Kremer et al. 2009). Thus such interaction may have a significant impact on embryonic development, since iron is essential in maturation and development of the insect eggs (Kurama et al. 1995). For example, the artificial overload of *Wolbachia* infection with *w*MelPop on double infections may cause excessive use of iron preventing normal deployment of host embryos, thus the resulting egg hatch can be dependent on differential iron level in host blood. Likewise, any nutritional competition between host and *Wolbachia* may cause insufficient provisioning of the embryo during development as suggested in the previous study (McMeniman et al. 2011).

The virulence of *w*MelPop appeared to decrease unlike the findings from a previous study (Suh et al. 2009). For example, egg hatch rates increased to that of the double infected *A. albopictus* in the later generations of the YFU population. Such results are similar to the virulence attenuation of *w*MelPop described in *Drosophila melanogaster* (McGraw et al. 2002). The increased egg hatch in YFU may occur because selection may have led to fixation of host genes that are more resistant to *w*MelPop as observed in *D. melanogaster* (Carrington et al. 2009). However, no changes in egg hatch were observed in *A. albopictus* that were singly infected with *w*MelPop that had utilized mouse blood (Suh et al. 2009), and it is unclear why the similar selection has not been observed with mouse blood.

Blood type affected maternal inheritance of the *w*MelPop infection in association with paternal type. Loss of the *w*MelPop infection was associated with mouse blood, particularly in the closed population, which is consistent with the prior study (Suh et al.

2009). Possible explanation is that *w*MelPop infected individuals are less fit than uninfected individuals when provided with mouse blood. As suggested above, if the embryos did not properly develop, they may suffer higher mortality during larval stage even after they survive to hatch. Consequently, proportion of uninfected larvae increases within the population and the infection frequency decreases when larvae develop to adult stage. However, existence of uninfected individuals that out-competed infected larvae still needs to be explained.

If the cost of infection is confirmed to be minimal with human blood comparing to other animal blood such as mouse blood, *w*MelPop may have potential application in controlling transmission of zoonotic pathogens vectored by *A. albopictus*. As the *w*MelPop spreads into the wild population, human blood feeding mosquitoes could have a higher reproductive output compared to conspecifics that fed on non-human animals. Thus, the *w*MelPop infection is more likely to spread in an anthropophilic population relative to a zoophilic population.

Double infection is frequently detected in a number of insect species (Rousset and Solignac 1995, Wenseleers et al. 1998, Van Borm et al. 2001, Malloch and Fenton 2005, Ponlawat and Harrington 2007, de Souza et al. 2009) while natural triple infection is relatively rare (Vavre et al. 1999, Kondo et al. 2002, Nirgianaki et al. 2003). Although artificial generation of the triple infection was not impossible, only two studies were reported in *Drosophila simulans* (Rousset et al. 1999) and *A. albopictus* (Fu et al. 2010). One possible reason for rare triple infections is that multiple infections may result in a fitness cost to the hosts, which counteract a fitness advantage from CI. Another is that triple infection is unstable due to resource competition among *Wolbachia* strains resulting

in reduced maternal inheritance of one or more *Wolbachia* strains during oogenesis in females. Considering the high density of *w*MelPop observed in the embryo of *A. albopictus* (Suh et al. 2009), *w*AlbA would be easily lost since relatively lower maternal inheritance of *w*AlbA has been observed in double infected *A. albopictus* (Kittayapong et al. 2002a). Comparing to the out-crossed population, the triple infection was close to 100% infection frequency in the closed population with human blood. This is presumably due to effective sterilization of CI inducing YFU males on single or double infected females (i.e., any individual that had lost *w*MelPop or/and *w*AlbA) preventing spread of single and/or double infection within the population.

The phenotypic effects of *w*MelPop infection appear to be additive regarding host longevity and the level of CI. Although the effect of life-shortening was not as strong as that shown in *w*MelPop infected *A. aegypti* (McMeniman et al. 2009), the reduced longevity was consistently observed in *w*MelPop infected females comparing to *w*MelPop-free individuals as shown in the prior study (Suh et al. 2009). The level of CI induced by the triple infection was similar to the observation in the single *w*MelPop infection in *A. albopictus* showing a 15~20% egg hatch rate in the incompatible crosses, confirming the additive characteristic of CI induction as found in the similar study of *A. albopictus* (Fu et al. 2010). However, no significant effect of *w*MelPop infection was observed on the egg viability over time unlike the previous study (McMeniman and O'Neill 2010), suggesting that the differential underlying mechanisms of phenotypic effect by *w*MelPop infection in embryonic stage need to be demonstrated.

Here in this study, phenotype of *w*MelPop infection was additive in *A. albopictus* by presenting life-shortening effects and unidirectional CI against double *Wolbachia*

infection. The association between host and the triple *Wolbachia* infection appears to develop from pathogenic toward mutualistic unlike the initial observation in single *w*MelPop infected *A. albopictus* (Suh et al. 2009). More embryos survived from virulence of *w*MelPop in compatible crosses, resulting in a reproductive advantage on triply infected *A. albopictus* by utilizing human blood source. Thus, employment of the triple *Wolbachia* infection in the population replacement strategy may require careful examination on host utilization of mosquitoes on target study areas, which may affect the spread of the triple *Wolbachia* infection in the natural population of *A. albopictus*. The tendency to favor human blood in triply infected *A. albopictus* will be particular interests in developing control strategies for animal diseases vectored by *A. albopictus*.

**Table 3.1.** Survival and infection status of *A. albopictus* microinjected with *w*MelPop *Wolbachia*

| Expt. | % hatch rate (larvae/injected eggs) | % pupation (pupae/larvae) | % eclosion (adult/pupae) | $G_0$ infection status (% infected) | |
|---|---|---|---|---|---|
| | | | | Female (infected/ total tested) | Male (infected/ total tested) |
| 1 | 1 (1/109) | 100 (1/1) | 100 (1/1) | NA (0/0) | NA (0/0) |
| 2 | 11 (9/82) | 100 (9/9) | 67 (6/9) | 100 (2/2) | 100 (4/4) |
| 3 | 8 (9/119) | 89 (8/9) | 100 (8/8) | 0 (0/4) | 67 (2/3) |
| 4 | 4 (5/120) | 100 (5/5) | 100 (5/5) | 67 (2/3) | 50 (1/2) |
| 5 | 0 (0/107) | NA (0/0) | NA (0/0) | NA (0/0) | NA (0/0) |

NA = not applicable.

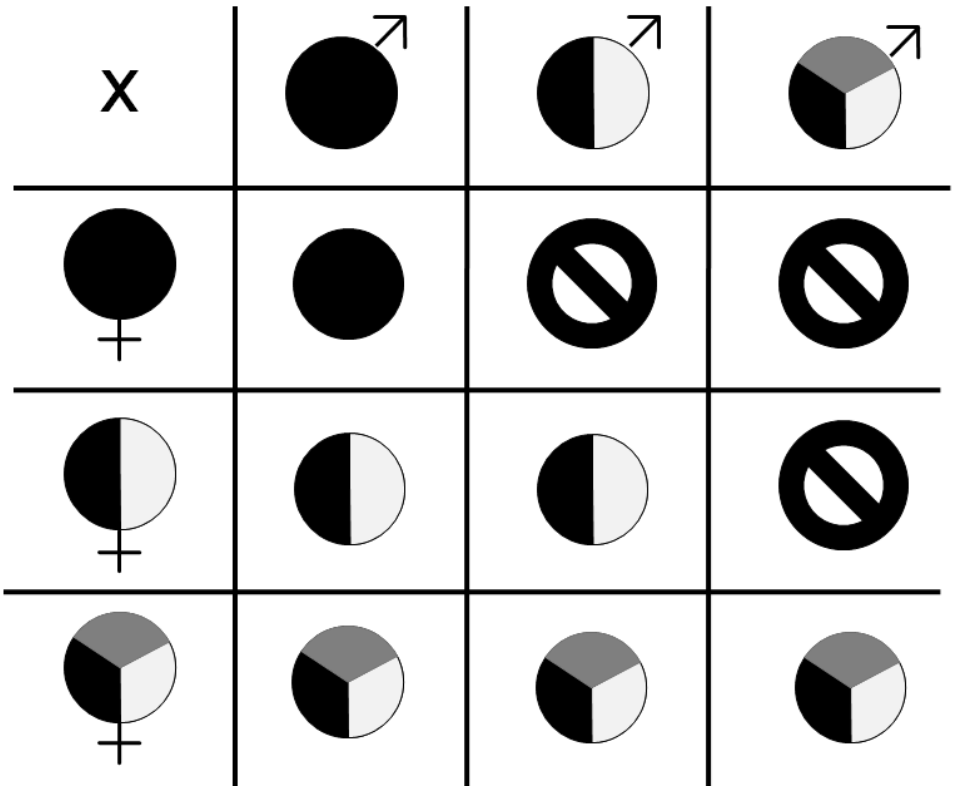


**Figure 3.1.** Results of mating between females and males harboring different strains of *Wolbachia* represented as different color. If the females do not harbor *Wolbachia* strains from the paternal type, the cross is incompatible resulting in no or reduced number of hatching broods (crossed circle). Other crosses are compatible and produce normal progeny inheriting maternal infection type.

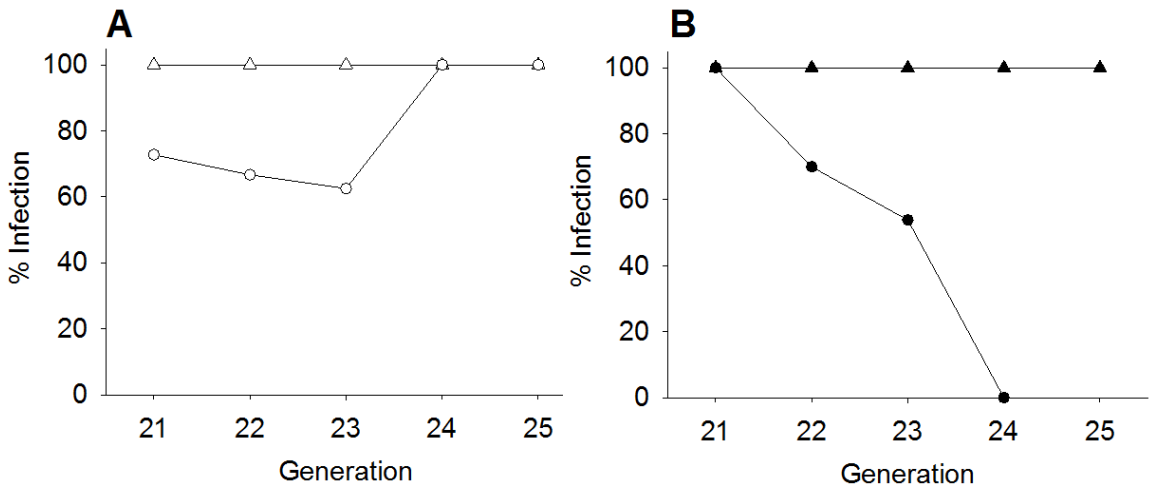


**Figure 3.2.** Infection frequency of *w*MelPop among progeny of YFU females mated with UT males (A: out-crossed population) and YFU males (B: closed population) provided with human blood (triangle) and mouse blood (circle). No loss of *w*AlbA and *w*AlbB was detected during the experiment.

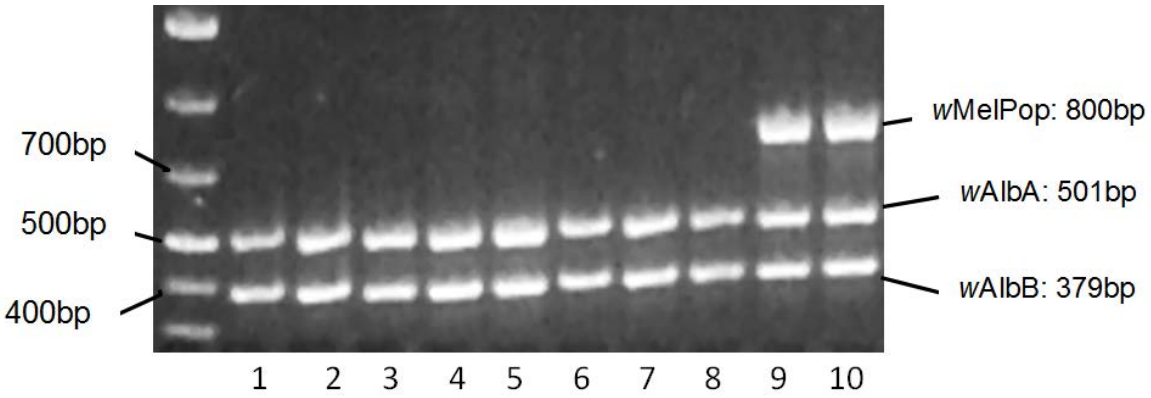


**Figure 3.3.** No evidence of paternal inheritance of *w*MelPop from incompatible cross between triple infected YFU males (i.e., infected with *w*MelPop, *w*AlbB and *w*AlbB) and double infected IH females (i.e., infected with *w*AlbB and *w*AlbB). Lane 1 and 2 = *w*MelPop negative controls, 20 pooled L1 from IH × IH (female × male) per sample. Lane 3-8 = test samples, 20 pooled L1 from IH × YFU per sample. Lane 9 = positive control 1 (one L1 from YFU × IH plus 19 L1 from IH × IH). Lane 10 = positive control 2 (two L1 from YFU × IH plus 18 L1 from IH × IH).

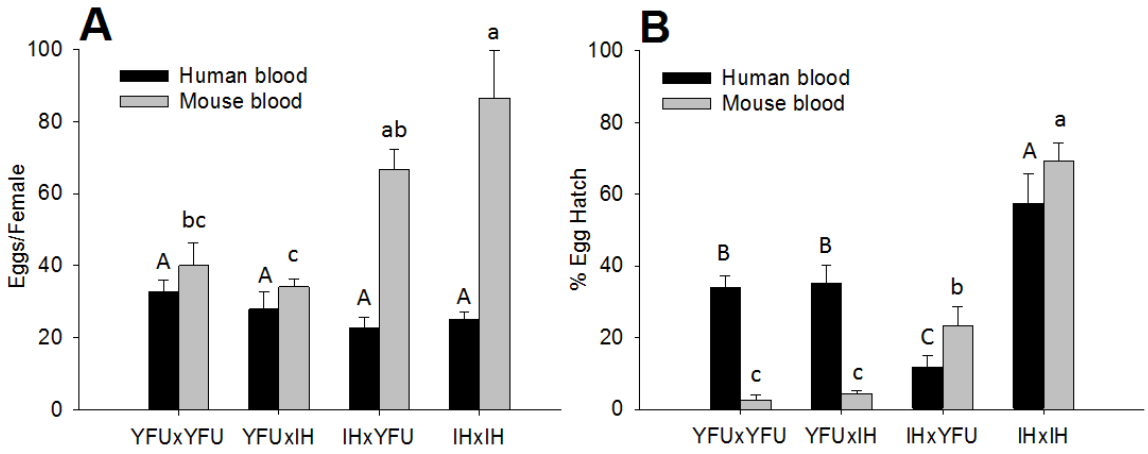


**Figure 3.4.** Fecundity (A) and egg hatch (B) resulting from crosses (female × male) between IH (double-infected *A. albopictus*) and YFU (*w*MelPop *Wolbachia* infected IH), providing human and mouse blood. Different letters represent significant difference at *P* = 0.05. Error bar = SE (n = 5).

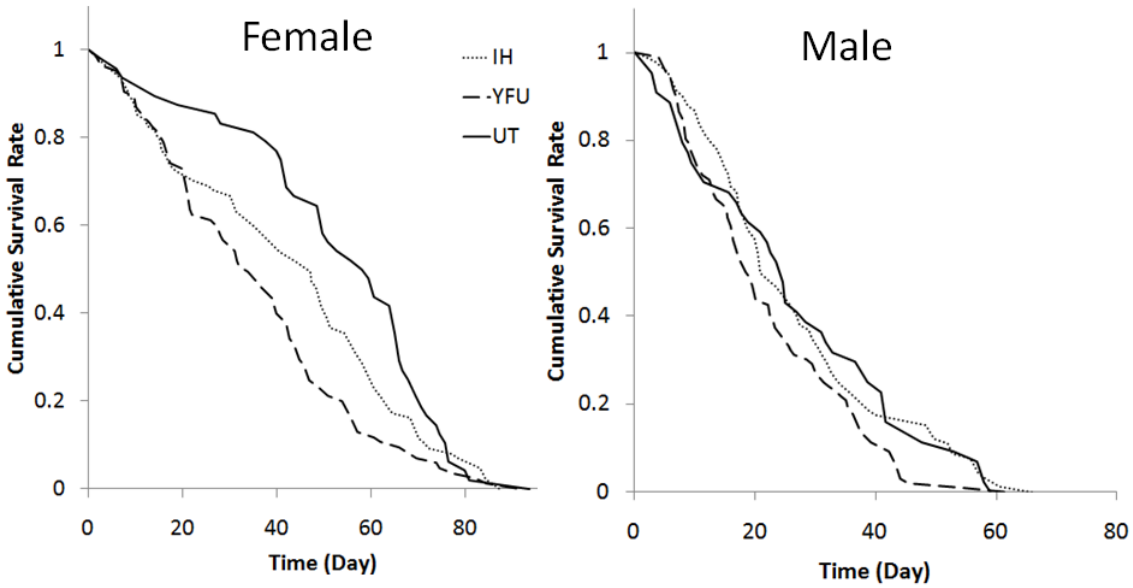


**Figure 3.5.** Adult longevity of IH (double-infected *A. albopictus*; dotted line), YFU (*w*MelPop infected IH; dashed line) and UT (uninfected *A. albopictus*; solid line).

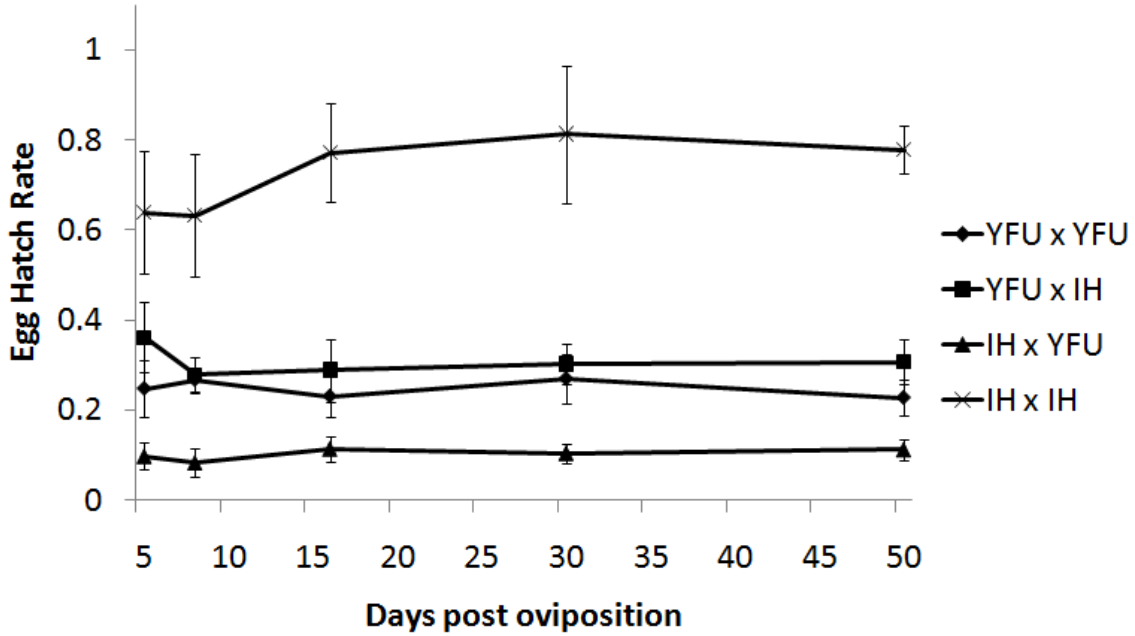


**Figure 3.6.** Viability of eggs resulting from crosses between IH (double-infected *A. albopictus*) and YFU (*w*MelPop *Wolbachia* infected IH) (female × male). Error bar = SE (n = 5).

# Chapter Four

## Characterization of life-shortening *Wolbachia* in *Aedes aegypti*

### Introduction

*Aedes aegypti* is a primary vector of dengue viruses. The breeding sites of *A. aegypti* are in close proximity to human residences thus the mosquito often blood feeds on humans becoming an efficient disease vector (Harrington et al. 2001, Wilder-Smith and Gubler 2008). There are an estimated three billion people at risk of dengue fever or dengue hemorrhagic fever, most of whom live in tropic and subtropic areas (Gubler 2002). With no registered vaccines, efforts to limit dengue virus transmission are mainly dependent on vector control, which often fail to provide sustainable control methods. Thus, complementary strategies are proposed and being development, including utilization of *Wolbachia*.

*Wolbachia* are maternally inherited intracellular bacteria identified in a wide range of invertebrate species (Hilgenboecker et al. 2008, Werren et al. 2008). In order to persist and spread in hosts, *Wolbachia* has evolved to form parasitic to mutualistic associations with hosts. Examples of mutualism are observed in obligatory associations where *Wolbachia* is essential for host reproduction (Bandi et al. 1999, Hoerauf et al. 1999, Dedeine et al. 2001) and facultative associations, where *Wolbachia* have direct positive effects on host fitness by increasing fecundity (Vavre et al. 1999), male fertility (Wade and Chang 1995), metabolic plasticity (Brownlie et al. 2009), immunity to pathogens (Teixeira et al. 2008), and/or multiple life history traits (Dobson et al. 2002b, Fry et al. 2004). On the other hand, parasitic *Wolbachia* can indirectly increase the relative host fitness through manipulation of host reproduction including feminization,

parthenogenesis, male killing, and cytoplasmic incompatibility (CI) (Werren et al. 2008). However, such *Wolbachia* infections typically impose physiological cost due to metabolic competition (i.e., nutrition use by *Wolbachia*) resulting in decreased host fitness (Hoffmann et al. 1990, Fleury et al. 2000, Perrot-Minnot et al. 2002 , Huigens et al. 2004, Rigaud and Moreau 2004, Duron et al. 2006, Islam and Dobson 2006). Thus, the infection dynamics of *Wolbachia* depends on interactions between the cost of infection and the relative fitness advantage obtained from *Wolbachia* infection.

A life-shortening strain of *Wolbachia* has been isolated from *Drosophila melanogaster* (Min and Benzer 1997 ), acclimated in mosquito cells (designated as *w*MelPop-CLA: *w*MelPop cell-line-adapted strain of *Wolbachia*) (McMeniman et al. 2008) and introduced into *A. aegypti* (McMeniman et al. 2009). The observed phenotype was strong CI and a reduction of longevity in adult mosquitoes when compared to uninfected strains. Egg hatch was 0% when uninfected females mate with infected males, while infected females normally produced progeny. Thus, the infected host is expected to have a relative fitness advantage over the uninfected during reproduction in panmictic population. In this case, the results may lead to population replacement with individuals harboring the *Wolbachia* infection, as observed in the prior studies (Turelli and Hoffmann 1991, Xi et al. 2005a). Thus, the proposed strategy is to release the life-shortening *Wolbachia* infected individuals resulting in the invasion of the *Wolbachia* infection in the vector population, consequently skewing the age structure toward younger age classes. As a result of the population replacement, the probability for disease transmission can be reduced since female mosquitoes must survive an extrinsic incubation period (EIP) to transmit dengue or other pathogens. Additional studies have

also demonstrated that the *w*MelPop-CLA infection in *A. aegypti* was responsible for inhibiting pathogen proliferation in mosquitoes (Kambris et al. 2009, Moreira et al. 2009b). In addition to life-shortening effects, the refractory traits against diseases will potentially be advantageous in controlling disease transmission.

Important factors affecting invasion dynamics of *Wolbachia* include level of CI, maternal inheritance, and fitness costs on hosts (Hoffmann et al. 1990). In order for the infected mosquitoes to initiate invasion into the vector population, the released individuals must reach a critical threshold relative to the target vector population size. This particular ratio is called the 'unstable equilibrium point' where *Wolbachia* spread can be initiated leading to fixation, which is positively correlated with fitness cost of *Wolbachia* infection (e.g., adult life-shortening) (Turelli 2010). The effects of *w*MelPop-CLA in *A. aegypti* have been described for the key parameters in part, showing 100% CI and maternal inheritance, and no cost on fecundity and egg hatch rate (McMeniman et al. 2009).

Here, my study focuses on characterizing *w*MelPop-CLA infection to determine additional traits that are predicted to affect the relative fitness of infected *A. aegypti*. Experiments assess impacts of the *Wolbachia* infection on 1) intra-specific and inter-strain competition at immature stage with two larval densities, 2) mating competition of males, and 3) egg viability at two temperatures. Ultimately, this study provides further information that will help understanding infection dynamics of the *Wolbachia* and population dynamics of *A. aegypti*.

## Materials and Methods

**Insect strains.** Experiments used the *w*MelPop infected colony of *A. aegypti* (PGYP1) and an aposymbiotic *A. aegypti* (PGYP1.tet) strain that had been derived from PGYP1 by tetracycline treatment to remove the *Wolbachia* infection (McMeniman et al. 2009). All maintenance and experiments were conducted at 28 ± 2 ºC, 75 ± 10% RH, and a photoperiod of 18:6h (L:D). For routine maintenance of mosquito strains, eggs were submerged in a mixture of fish food (TetraMin Tropical Tablets, Tetra, Germany) in 400 ml of water. Larvae were given fish food and adults were transferred into 30 × 30 × 30cm cages with constant access to a 10% sucrose solution. The PGYP1 strain was blood fed with an artificial feeder and unexpired human blood from the Kentucky Blood Center (Lexington, KY). The PGYP1.tet strain was provided with anesthetized mice for blood feeding (PHS Assurance #A3336-01).

**Competitiveness of immature *Aedes aegypti*.** To determine optimal rearing conditions for development of immature *A. aegypti*, 300 larvae were reared with a series of different resource regimes. Fifty PGYP1 larvae (< 2h post egg hatch) were transferred into containers (Mosquito Breeders; BioQuip, Rancho Dominguez, CA) with 200ml water and were provided with 10, 40, 70, 100, 130 or 160mg fish food every third day until pupation. Eclosing adults were counted; their sex was determined, and their developmental time (time to emergence) was recorded.

To examine the competitiveness of the *Wolbachia* infected larvae relative to naturally uninfected *A. aegypti*, known numbers of PGYP1 and PGYP1.tet larvae (< 2h post hatch) were placed into containers and reared as described above. Larvae were fed with 70mg of fish food every third day. Two larval density conditions (low = 50 larvae;

high = 400 larvae) were compared. At each density, three ratios of infected:uninfected larvae were compared: 1:0, 'Infected' (I); 0:1, 'Uninfected' (U); and 1:1, 'Mixed' (M). Each of the six treatments was replicated four times. Eclosing adults were removed daily, until no viable immature individuals remained. The sex, eclosion time and wing length of emerging adults were recorded.

Wing size and *Wolbachia* infection status was determined for a subset of eclosing adults. To measure wing size, image of wings were captured using zPix MM-640 microscope (Carson Optical, Hauppauge, NY), and the wing length was estimated using ImageJ software (Barboriak et al. 2005). For PCR, DNA was extracted from adult mosquitoes as described previously (Brelsfoard et al., 2008). PCR amplification was performed in 25µl reaction volumes using Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) with 16s *Wolbachia* specific (Werren and Windsor 2000) and CO1 universal primers (Hebert et al. 2003), to determine both *Wolbachia* infection and template quality. 17.5µl of $H_2O$, 2.5µl of 10X buffer, 0.8µl of dNTP (10mM), 0.5µl of W-specf, W-specr, CO1f and CO1r each (10µM), 0.2µl Taq and 2µl of DNA template. A MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used to perform 94°C, 2 min and 38 cycles of 94°C, 2 min; 55°C, 45 sec and 72°C, 1.5 min followed by 72°C, 10 min.

Although all eclosing adults were collected, a sampling approach was used to assess size and infection status. To avoid a sampling bias, females and males were each divided into five equal size groups, according to eclosion time. For the low density I and U treatments, five females and five males were randomly selected from each of the resulting groups. In an attempt to collect a similar number of infected/uninfected

individuals from the M treatment, the sample size was doubled (i.e., ten females and ten males from each group). For the high density condition (HD), a similar sampling approach was used, but the sample size was increased by four times in each treatment.

Survival was calculated as the number of collected adults divided by the number of larvae initiated for the experiments. To estimate the relative competitiveness of infected and uninfected individuals within the M treatment, the following equations were used to estimate the ratio of infected: uninfected individuals for each sex. $E_{fi}$ is the estimated proportion of infected females (i.e., the proportion of females infected multiplied by the sex ratio), expressed as:

$$E_{f_i} = \left(\frac{f_i}{f_i + f_u}\right)\left(\frac{f}{f+m}\right) \quad \text{Equation 1}$$

where $f_i$ and $f_u$ are the number of infected and uninfected females resulting from the PCR test, respectively; $f$ and $m$ are the total number of eclosing females and males, respectively. A similar method was used to estimate the proportion of uninfected females ($E_{f_u}$), infected males ($E_{m_i}$) and uninfected males ($E_{m_u}$). Assuming equal sex ratio and survival of infected and uninfected individuals, the proportion of each would be: $E_{f_i} = E_{f_u} = E_{f_u} = E_{f_u} = 0.25$. Thus, the departure from equal emergence for infected females ($D_{f_i}$) was calculated as $4E_{f_i} - 1$, and similar calculations were made for uninfected females ($D_{f_u}$), infected males ($D_{m_i}$) and uninfected males ($D_{m_u}$). Estimated ratios for infected and uninfected individuals were compared using *t*-test after arcsine transformation to compare relative survival by sex for each larval density.

To examine for an effect of condition (i.e., LD or HD) and/or infection status on survival or sex ratio, generalized linear models were used (binomial distribution with

Logit link; JMP 8.0.1; SAS Institute, Cary, NC). Chi-square tests (two-tailed Fisher's exact) were used to compare survival. Two-way ANOVA tests were used to examine for an effect of larval density condition and sex on mean development time or size. One-way ANOVA tests were used to compare mean development time and size among individuals emerging from the infected (I), uninfected (U) and mixed (M) treatments. For these comparisons, individuals from the mixed (M) treatments were divided into M+ (infected individuals in the M treatment) and M- (uninfected in the M treatment).

To examine the combined impact of *w*MelPop infection on survival, developmental time and resulting fecundity, a simplified index of performance (*I*) was calculated simulating per capita rates of change following a previous study (Koenraadt et al., 2010).

$$I = \frac{\ln \frac{1}{N_0} \sum_x A_x \bar{w}_x}{\sum_x x A_x \bar{w}_x \Big/ \sum_x A_x \bar{w}_x}$$ Equation 2

where $N_0$ is the initial number of females (assumed to be 50% of the initial immature number in the experiments), $A_x$ is the number of adult females produced at time $x$ , $\bar{w}_x$ is the mean size of the emerging females. Indices of performance were calculated from all emerging females to examine the effects of condition or infection status on the performance using two-way ANOVA after log transformation. To understand relative performance among the treatments, the mean index value of U treatment of low density condition was set at 1, as the reference group.

Within the mixed treatment (M), the relative performance of infected (M+) and uninfected (M-) immature individuals were compared. Indices of performance were

calculated from sampled individuals determined to be infected (M+) and uninfected (M-) to examine the effects of condition and/or infection status on the performance using two-way ANOVA after log transformation. To understand relative performance of M+ and M- treatments, the mean index value of the M- treatment was set at 1 of low density condition as the reference group within each condition.

**Male mating competitiveness.** Adult males of the PGYP1 and PGYP1.tet strains were compared to examine for a hypothesized difference in male mating competitiveness. Twenty virgin PGYP1 females (< 2 days old) were mated with differing ratios of PGYP1 and PGYP1.tet males (40:0, 30:10, 20:20, 10:30 and 0:40; < 2 days post eclosion). After blood feeding on human blood, engorged females were transferred into individual oviposition cups and allowed seven days to lay eggs. Mating competitiveness was examined by comparing observed and predicted ratio of hatching brood per cage using Chi-square tests (two-tailed Fisher's Exact; JMP 8.0.1; SAS Institute, Cary, NC). Broods resulting in lower than 18% egg hatch (the lowest hatch rate observed in the compatible crosses) ware scored as incompatible crosses. To examine for multiple mating (i.e., females using sperm from multiple males), egg hatch from compatible crosses were compared among the differing ratios of incompatible males using Kruskal-Wallis test. To examine for an effect of increasing ratio of incompatible males on the egg hatch rate of PGYP1 females, pair-wise Man-Whitney tests were used.

**Egg viability.** The effect of *w*MelPop-CLA infection on the viability of *A. aegypti* egg was examined in association with egg age and temperature. PGYP1 and PGYP1.tet females were blood-fed with human blood, and eggs were collected for five days. Collected eggs were stored at 100% RH for 3 days and 75% RH for 2 days. The resulting

egg papers were divided into eight parts and stored at 15°C and 28°C with constant 70% relative humidity. Eggs were hatched at eight time points between six and 40 days after oviposition. Eggs were hatched for three days and fish food was provided *ad libitum*. Multiple linear regression analysis was used to determine trends in egg viability of each strain over time at two different temperatures (JMP 8.0.1; SAS Institute, Cary, NC). In order to compare overall mean egg hatches, Friedman test (nonparametric repeated measures ANVOA) was conducted between strains and temperatures (SPSS 17.0; SAS Institute, Cary, NC).

## Results

### Larval competitiveness

**Range finding.** From the range finding test, 70mg food was selected as a minimal food amount that resulted in high survival and short development time of 50 larvae, and this food level was used subsequently in larval competition assays (Fig. 4.1).

**Survival.** At the LD condition (50 larvae), PGYP1.tet showed higher survival (U treatment; 95.5 ± 1.7%; mean ± SE) compared to PGYP1 larvae (I treatment; 87.0 ± 1.3%) (Fig. 4. 2A) (Chi-square test; $P < 0.01$). In contrast, no difference was observed in survival between PGYP1 (I treatment; 79.3 ± 0.9%) and PGYP1.tet (U treatment; 78.5 ± 1.5%) at HD condition ($P = 0.6$). Significant effect of condition, infection status and the interaction of condition × infection status were observed (Table 4.1).

**Sex ratio.** The sex ratio calculated by female proportion among eclosed adults did not differ significantly between PGYP1 and PGYP1.tet regardless of larval density (Fig. 4.2).

Generalized linear model did not observe the effect of larval density condition or infection status ($\chi^2 = 7.23$, df = 3, $P = 0.065$).

**Relative survival in mixed treatment**. Relative survival of infected and uninfected individuals from the M treatment was not different for either females ($F_{1,6} = 0.0084$, $P = 0.930$) or males ($F_{1,6} = 2.73$, $P = 0.149$) at LD condition (Fig. 4.3). In contrast, infected females were observed to have a significantly lower survivorship than uninfected females ($F_{1,4} = 40.55$, $P < 0.01$) at the HD condition. No difference was observed in the male survival ($F_{1,4} = 3.28$, $P = 0.144$) in the HD condition.

**Development time.** Individuals from LD developed faster than those from HD condition for both sexes (two-way ANOVA; $F = 2798$, df = 1, $P < 0.0001$), and males developed faster than females ($F = 35.9$, df = 1, $P < 0.0001$). At LD condition, no difference was observed between infected (I) and uninfected females (U) in the mean developmental time (Fig. 4.4). In the mixed treatments (M), the infected individuals (M+) developed slower than the uninfected (M-) when they competed directly, for both females (one-way ANOVA; $F_{3,12} = 9.31$, $P < 0.01$) and males ($F_{3,12} = 8.03$, $P < 0.01$). The same pattern was observed in HD for females ($F_{3.10} = 7.12$, $P < 0.01$) and males ($F_{3.10} = 4.53$, $P < 0.05$). When the development time of infected (I) and uninfected individuals (U) were compared without M+ and M- treatment, significant difference was observed with the pattern that infected individuals developed slower than the uninfected, except for the females at HD condition.

**Size.** Using wing length as a determinant for adult size, emerged individuals from the LD condition were larger than those from HD in both sexes (two-way ANOVA; $F = 1885$, df = 1, $P < 0.0001$), and females were larger than males ($F = 1716$, df = 1, $P < 0.0001$).

At the LD condition, no difference was observed among four treatments (I, U, M+ and M-) in both females (one-way ANOVA; $F_{3,12} = 2.95$, $P = 0.0758$) and males ($F_{3,12} = 3.20$, $P > 0.0624$) (Fig. 4.5). At HD condition, a pattern was observed that the uninfected females (U) were larger than the infected (I) but the difference became insignificant when they competed directly each other within the M treatment ($F_{3,10} = 7.12$, $P < 0.01$). Infected (I) and uninfected males (U) were not different in size, but infected males (M+; 1.71 ± 0.0036mm; mean ± SE) were larger than the uninfected (M-; 1.65 ± 0.0092 mm) when they competed directly ($F_{3,10} = 4.53$, $P < 0.05$).

**Index performance.** The index performance was higher in LD than HD whether infected and uninfected individuals were reared in separate containers (two-way ANOVA; $F = 413$, df = 1, $P < 0.01$), or in the same container ($F = 182$, df = 1, $P < 0.0001$) (Fig. 4.6). No effect of infection status on performance index was observed when infected and uninfected were reared in separate container ($F = 3.9$, df = 1, $P > 0.05$). In contrast, a significant effect of infection status ($F = 12.09$, df = 1, $P < 0.01$) and infection × condition interaction ($F = 5.52$, df = 1, $P < 0.05$) on performance index was observed when infected and uninfected were reared in the same containers. Particularly, a significant difference was observed at HD condition between M+ and M- by 48% (*t*-test; $F_{1,4} = 68.53$, $P < 0.01$)

**Mating competitiveness**

Chi-square test did not detect difference between predicted and observed egg hatch ($P > 0.5$) indicating no difference in mating competitiveness between infected and uninfected males. Egg hatch of PGYP1.tet decreased with an increased number of incompatible PGYP1 males, following a linear relationship ($R^2 = 0.9884$) (Fig. 4.7). A significant

difference was observed in the egg hatch of all broods among the cages containing different ratios of incompatible males (Kruskal-Wallis; $\chi^2 = 27.9$, df = 4, $P < 0.0001$), with the pattern that the egg hatch decreased as the proportion of incompatible males increased in the cages. To examine potential multiple mating in PGYP1.tet females, the egg hatch of compatible broods were compared, resulting in no difference ($\chi^2 = 0.98$, df = 3, $P = 0.81$).

**Egg viability**

*w*MelPop-CLA infection significantly decreased viability of eggs of *A. aegypti* over time and the reduction was pronounced at higher temperature. Viability of uninfected eggs of *A. aegypti* was constant over time at both 15°C ($R^2 = 0.021$, $F_{1, 38} = 0.80$, $P = 0.38$) and 28°C ($R^2 = 0.018$, $F_{1, 38} = 0.68$, $P = 0.42$) (Fig. 4.8). In contrast, viability of infected eggs decreased over time at both 15°C ($R^2 = 0.92$, $F_{1, 38} = 431.96$, $P < 0.0001$) and 28°C ($R^2 = 0.88$, $F_{1, 38} = 297.52$, $P < 0.0001$). A Friedman test compared mean egg hatches combined from all time points resulting in significant difference among each strain at different temperatures ($\chi^2 = 104.73$, df = 3, $P < 0.0001$). Pair-wise comparison using a Friedman test revealed that egg hatches of PGYP1.tet were not significantly different between 15°C (92.78 ± 0.66%; mean ± SE) and 28°C (92.30 ± 0.51%) treatments ($\chi^2 = 5.77$, df = 1, $P = 0.096$; Bonferroni corrected). In contrast, a significant difference was observed in the egg hatch of PGYP1 between 15°C (46.38 ± 4.13%) and 28°C (39.25 ± 4.13%) treatments ($\chi^2 = 16.9$, df = 1, $P < 0.001$; Bonferroni corrected). Egg hatch of infected strains were lower than the uninfected strains regardless of temperature at the significance level of $P = 0.05$.

**Discussion**

Effects of *w*MelPop-CLA are best characterized as virulent *Wolbachia* causing reduced fitness of hosts due to life-shortening ability in adults. Such effects are consistently observed in other life stages, including immature stages (McMeniman et al. 2009). Previous reports have suggested that the underlying mechanism of life-shortening effects is due to the over-proliferation of *Wolbachia* in host cells (Min and Benzer 1997). Alternatively, the increase in the *Wolbachia* proliferation could have direct costs on host physiology by damaging host cells or tissues as observed in the prior studies (Min and Benzer 1997, McGraw et al. 2002, Reynolds et al. 2003, McMeniman et al. 2008) and/or causing potential nutrition competition between *Wolbachia* and host cells.

The *w*MelPop-CLA infection caused reduced relative competitiveness in immature individuals. The negative impacts of the infection were more pronounced as competition effects increased between infected and uninfected immature individuals with limited resources. The detrimental effect of *Wolbachia* infection is consistent with the prior study that naturally *Wolbachia* infected *A. albopictus* suffered higher mortality and longer developmental time from direction competition with uninfected individuals when food stressed (Gavotte et al. 2010). However, the *Wolbachia* infection was beneficial for *A. albopictus* showing higher survival, reduced developmental time and increased sex ratio when food was not limited, which was not observed in this study. In addition to virulent effect of *w*MelPop-CLA, the contrasting results are presumably due to the artificial association between *Wolbachia* and *A. aegypti* that has not yet developed to commensal or mutualistic relationship. A further study would be to select for higher

survival of infected immature individuals, which may cause the virulence attenuation as observed in the prior study (McGraw et al. 2002).

Infected individuals appeared to obtain size advantage from direct competitions with uninfected individuals. Infected females that survived under strong intra-specific immature competition (i.e., high density condition) were smaller than uninfected females but the difference became insignificant when infected and uninfected individuals competed directly. A similar pattern was observed for the infected males, which infected males were larger than uninfected males. Possibly, late developing larvae (i.e., infected larvae) could consume more food per larva as larvae eclosed or died from the competition, which is thus a tradeoff between development time and size. Size is positively correlated with female fecundity (Naksathit and Scott 1998, Blackmore and Lord 2000). Also, larger males tend to increase reproductive potential by producing larger amount of sperm for mating (Ponlawat and Harrington 2007, 2009).

Larval competition has been suggested as a key factor that determines invasion ecology in mosquitoes (Livdahl and Willey 1991). As an example, superior competitiveness of *A. albopictus* in the larval stage was identified to account for an extinction of cohabiting *A. aegypti* (Juliano 1998, Juliano and Lounibos 2005). In my study, the relative index of performance was used to examine a possible outcome of competition between infected and uninfected individuals, by combining the differential impact of *Wolbachia* infection and environmental conditions on female survival, developmental time and fecundity to compare relative growth rate of infected and uninfected population. In the low larval density condition, the similar size of infected females compared to uninfected females (i.e., equal fecundity) appeared to compensate

the delay in developmental time in infected females, resulting in no difference in reproductive potential between infected and uninfected individuals. In contrast, the index performance was significantly reduced for infected population when the competition effects were higher. The results can be explained by the increased mortality and developmental time while the infected females failed to obtain the size advantage. Therefore, infected individuals will be unlikely to invade or coexist with uninfected population in a food stressed condition. Indeed, when infected individuals are released in the field as a part of population replacement strategy, resources are likely to decrease and the larval competition effects will tend to increase in the natural habitats.

When infected individuals begin to colonize an uninfected population, infected and uninfected males will compete for wild type females for mating. If the *Wolbachia* infection disturbs male mating behavior, the proportion of females that have mated with incompatible males will decrease and subsequently delay the population replacement process. The results from the mating competition experiments observed here were consistent with prior studies showing no effects of *Wolbachia* infection on male mating competition in *A. polynesiensis* (Brelsfoard et al. 2008). As the deleterious effect of *w*MelPop-CLA was detectable mostly in older hosts (Min and Benzer 1997, McMeniman et al. 2009, Moreira et al. 2009a, Turley et al. 2009), no such effects were observed in the males that were less than three days old in my experiment. Thus, studies on potential *Wolbachia* effects on older males in mating competition can be useful for a further study. In addition, the results also suggest that there's no evidence of multiple mating (inseminated females utilize multiple sperm of different males), which could complicate measuring the sterilization effects of incompatible males on uninfected females.

Reduced embryo viability is analogous to the previous report that infected embryos had decreasing viability over time while egg hatch of uninfected embryos were relatively constant (McMeniman and O'Neill 2010). A decrease in egg hatch was not observed in naturally super infected *A. albopictus* (*w*AlbA and *w*AlbB) (Ruang-areerate et al. 2004) or artificially infected *A. aegypti* (Xi et al. 2005a). The egg viability was more reduced when eggs were exposed to higher temperature in this study. This observation is consistent with the previous results that adult mortality of *w*MelPop-CLA infected *A. aegypti* was dependent on temperature (McMeniman et al. 2009). Larval breeding sites in dry season tend to be void of water until the next rainfall, thus remain in a desiccation state. Mathematical models have suggested that reduced egg viability due to *Wolbachia* infection can dramatically decrease the probability of *Wolbachia* invasion particularly in the dry season or even result in extinction of infected population (Turelli 2010, Yeap et al. 2011). Replacement strategies should consider changes in temperature in the target area and the effects of higher temperatures in regions without the dry season.

The observed association between *Wolbachia* and host in this study frequently falls upon the category of pathogenic (Suh et al. 2009) rather than a mutualistic interaction (Dobson et al. 2002b). Consistent with this hypothesis, the over-replicating nature of *w*MelPop-CLA appears to be affecting the host at all life stages in this study. The virulent effects of *Wolbachia* tend to be amplified depending on the environmental conditions causing reduced competitiveness of infected individuals particularly in the immature stages. In such cases, the release number of infected individuals will need to be adjusted accordingly to facilitate the spread of *Wolbachia* infection, particularly for an age structured system (Turelli 2010). Increasing the release number of female

mosquitoes can increase the risk of disease transmission and the nuisance of additional mosquitoes, which may not be accepted by a local community. In order to reduce the number of releasing mosquitoes and the probability of failure in the proposed strategy, biotic or abiotic factors should be carefully examined to understand how these factors could affect immature and adult competition and/or *Wolbachia* virulence to hosts. Particularly, since development of immature mosquito larvae is greatly affected by temperature, a further study should include the effects of temperature on the relative competitiveness of infected immature individuals. Alternatively, as no fitness cost is observed, *w*AlbB infected *A. aegypti* (Xi et al. 2005a) can be further studied for the population replacement (e.g., immature competition) since *w*AlbB infection was reported to inhibit pathogen infections such as dengue virus (Bian et al. 2010).

**Table 4.1.** Generalized linear model to examine an effect of larval density condition (low or high density) and/or infection status (infected or uninfected) on survivorship

| Factors | Survivorship | | |
|---|---|---|---|
| | df | $\chi^2$ | *P* |
| Condition | 1 | 45.2 | < 0.0001 |
| Infection status | 1 | 8.13 | < 0.01 |
| Condition × infection status | 1 | 9.7 | < 0.01 |

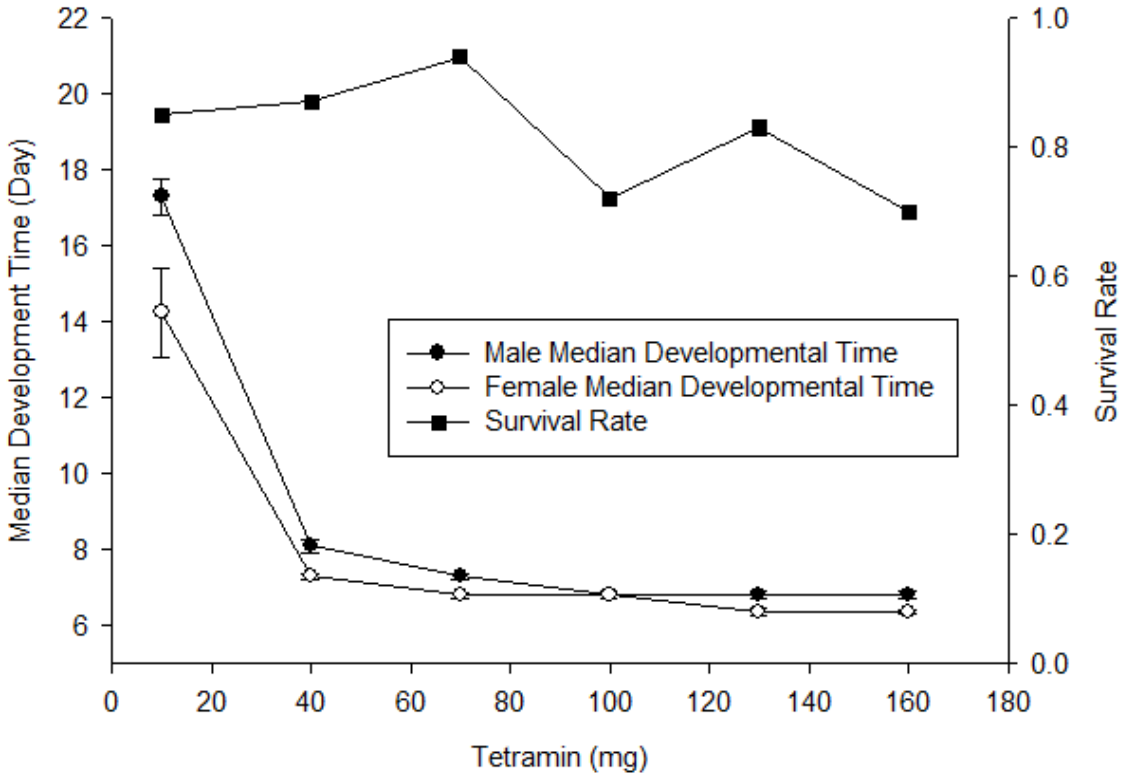


**Figure 4.1.** Median developmental time (Days) and survival rate [Total emerged/Initial number (50 larvae)] of *w*MelPop-CLA infected individuals with differing food amount. Error bar = SE.

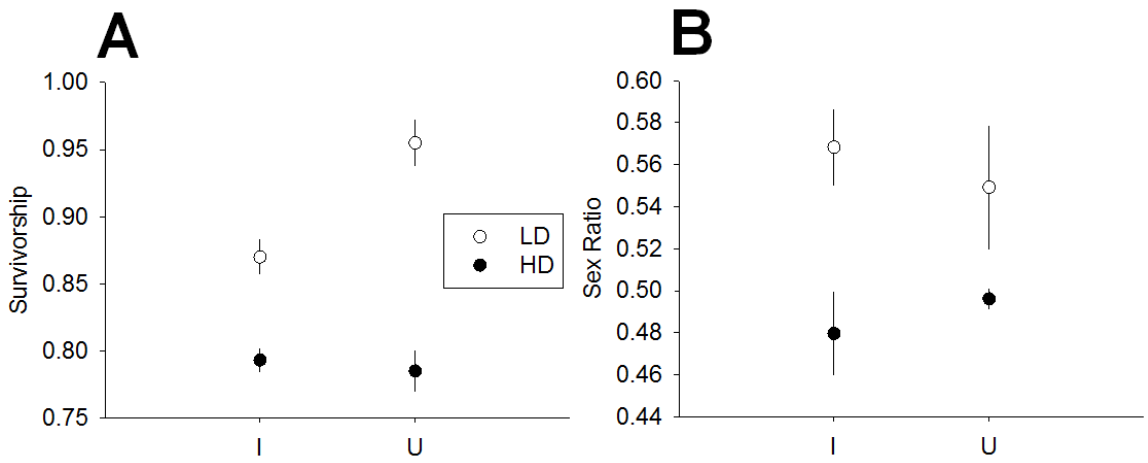


**Figure 4.2.** Survivorship (A) and sex ratio (B) of *w*MelPop-CLA infected (PGYP1, I treatment) and aposymbiotic *A. aegypti* (PGYP1.tet, U treatment) at two larval density conditions (LD, low density = 50 larvae; HD, high density = 400 larvae). Error bar = SE (n = 4).

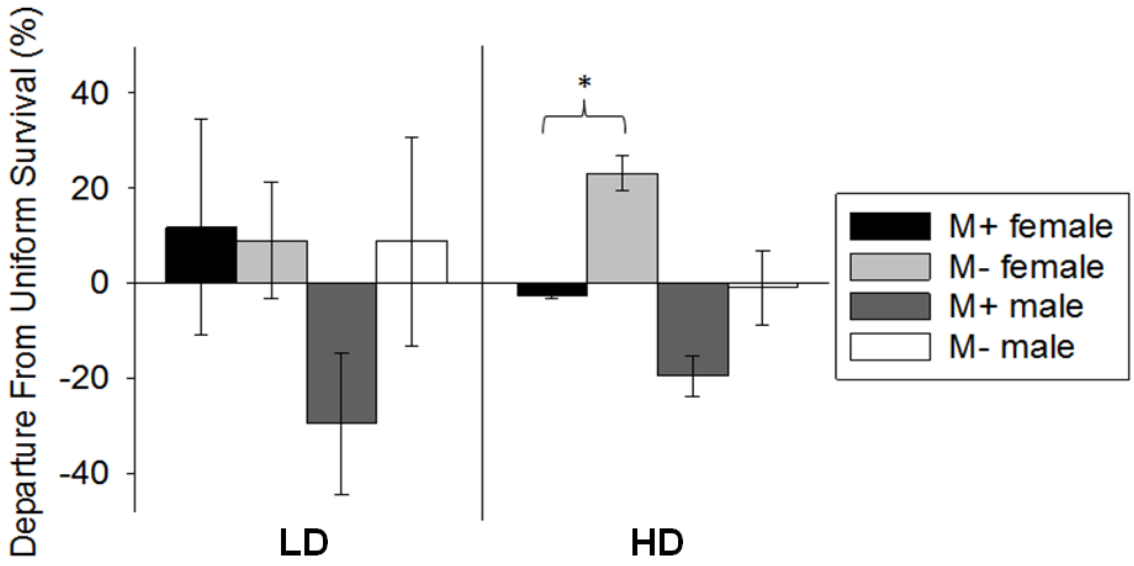


**Figure 4.3.** Departure from an equal emergence of *A. aegypti* competing at two larval densities (LD, low density = 50 larvae; HD, high density = 400 larvae). Asterisk represents significant difference at $P = 0.05$. Error bar = SE (n = 4).

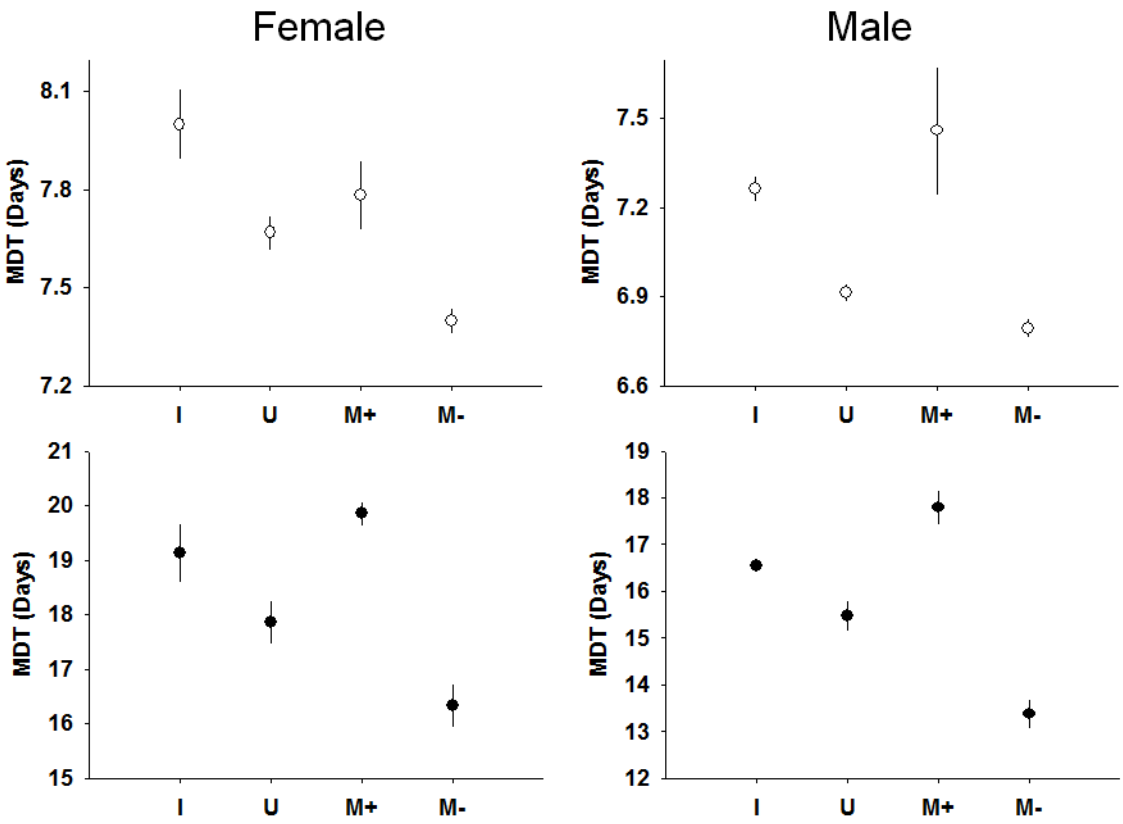


**Figure 4.4.** Mean development time (MDT) of *w*MelPop-CLA infected (PGYP1, I treatment), aposymbiotic (PGYP1.tet, U treatment), infected (M+) and uninfected (M-) in mixed population of *A. aegypti* at low (○; 50 larvae) and high (●; 400 larvae) larval densities. Error bar = SE (n = 4).

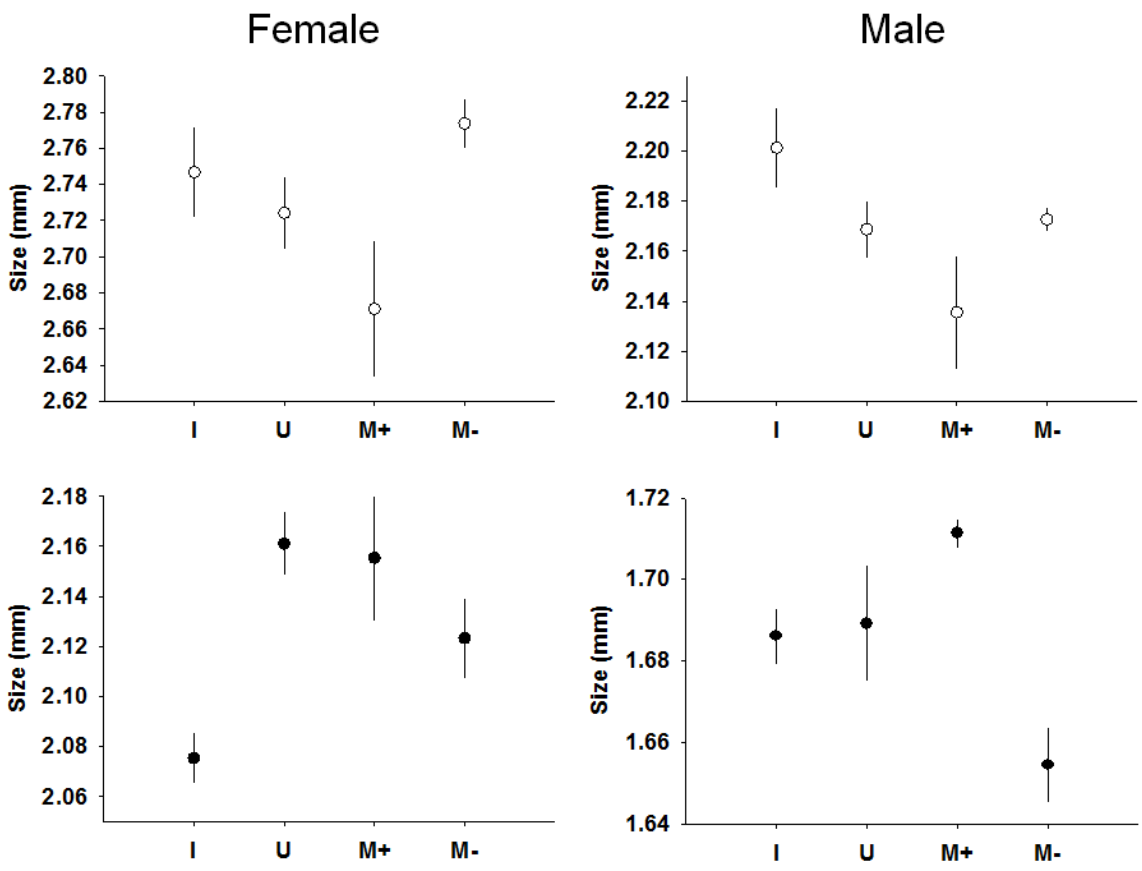


**Figure 4.5.** Wing size of *w*MelPop-CLA infected (PGYP1, I treatment), aposymbiotic (PGYP1.tet, U treatment), infected (M+) and uninfected (M-) in mixed population of *A. aegypti* at low (○; 50 larvae) and high (●; 400 larvae) larval densities. Error bar = SE (n = 4).

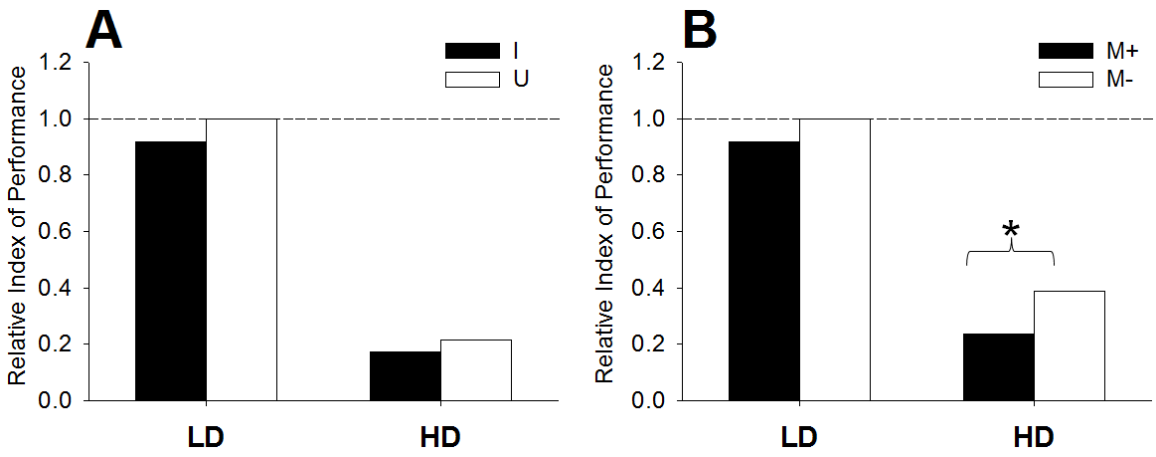


**Figure 4.6.** Relative index of performance of *w*MelPop-CLA infected (I), uninfected (U) treatments (A), and infected (M+) and uninfected (M-) within treatment of M (B) at two larval densities (LD, low density = 50 larvae; HD, high density = 400 larvae). Relative index of performance is an estimated rate of population growth. The horizontal dashed line represent standardized relative index (set as 1) for the uninfected treatments in low density condition, and the index performance of other treatments was calculated based on the standardized relative index. An asterix represents significant difference at $P = 0.05$.

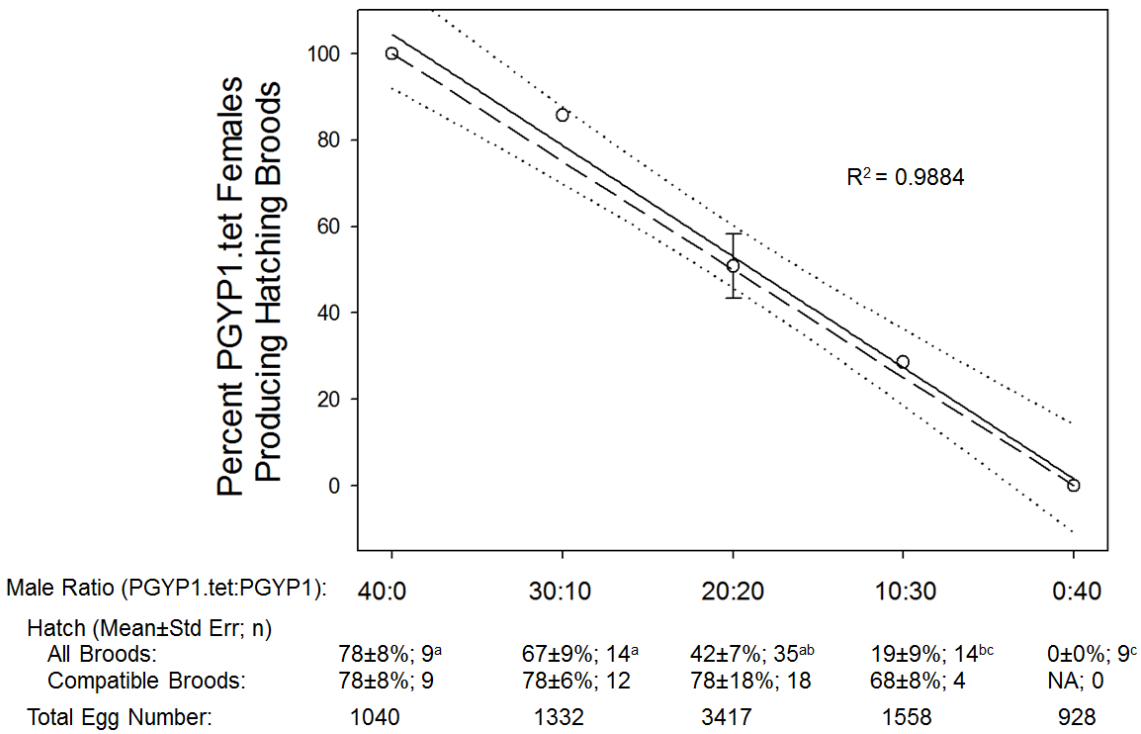


**Figure 4.7.** Male competitiveness of *w*MelPop-CLA infected *A. aegypti*. Based upon the observed egg hatch rate, females are scored as either 'compatible mating' ( = eggs hatching) or 'incompatible mating' ( = eggs not hatching). The percent compatible females (compatible females/total females) is determined for each cage replicate (20 females/cage; 3 cage replicates for 20:20 treatment). Circles and error bars indicate the mean ± standard error for each treatment (i.e., male ratio). The trend line (solid line) with 95% confidence intervals (dotted lines) is generated based upon the observed values. Predicted values (dashed line) are calculated assuming equal competitiveness of PGYP1.tet and PGYP1 males. Below the graph, egg hatch rates are based upon the combined oviposition of females from cages. Differing superscripted letters indicate significant differences (Man-Whitney test, $P < 0.05$, Bonferroni corrected). $R^2$ value is shown for the trend line fitted to observations.

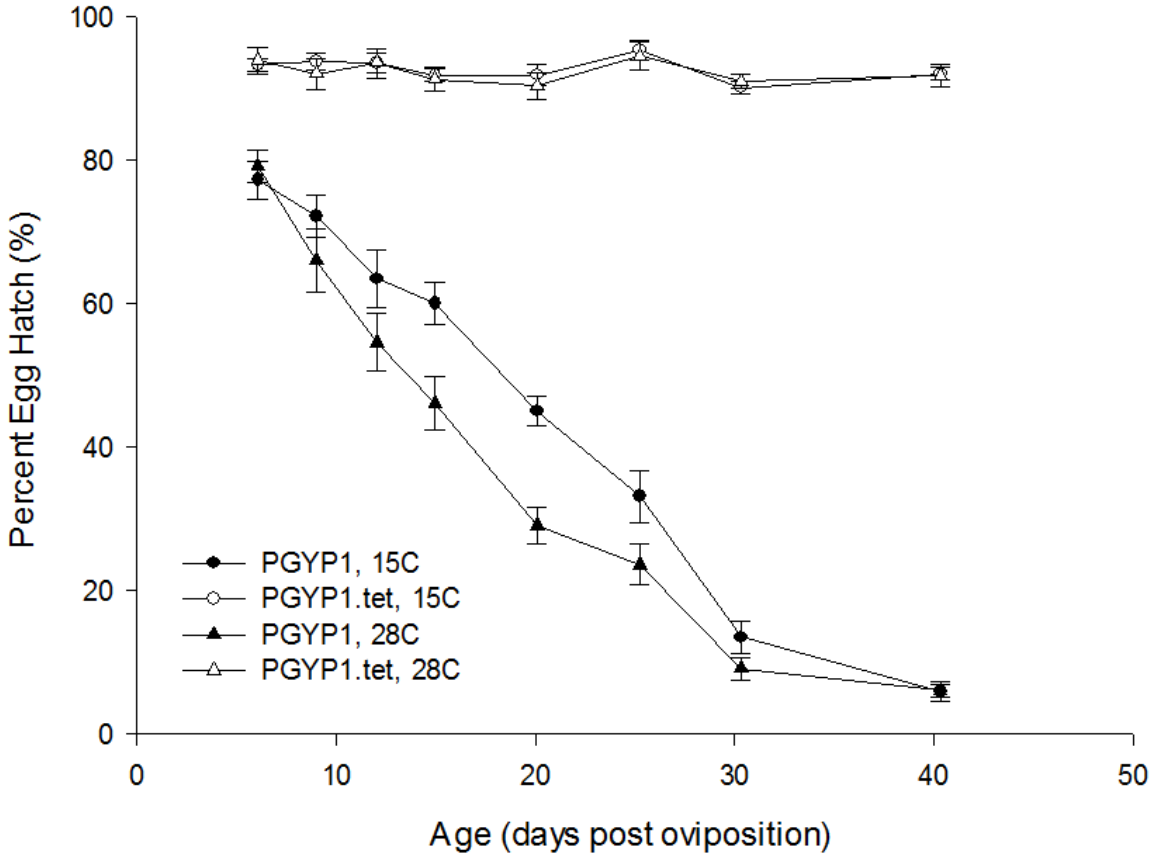


**Figure 4.8.** Viability of embryos from *w*MelPop-CLA infected (PGYP1) and uninfected (PGYP1.tet) *A. aegypti* over time at different temperatures with 70% relative humidity. Error bar = SE.

## Chapter Five

## *Wolbachia* effects on larval interactions in *Aedes aegypti*

### Introduction

*Wolbachia* is a maternally inherited endosymbiont commonly detected in a wide array of invertebrate species (Hilgenboecker et al. 2008). *Wolbachia* infections are responsible for a number of host reproductive manipulations including feminization, parthenogenesis, male killing or cytoplasmic incompatibility (CI) (Werren et al. 2008). These reproductive manipulations typically give *Wolbachia* infected individuals a reproductive advantage allowing for the spread and maintenance of *Wolbachia* in natural populations. CI is the most common reproductive manipulation described in insects and has received considerable attention as a method to control insect vectors and insect vectored disease (Bourtzis 2008).

The ability of *Wolbachia* to manipulate host reproduction has been highlighted in the development of *Wolbachia*-based disease control strategies such as population replacement. The costs and benefits of *Wolbachia* infections are now of growing interest in characterizing *Wolbachia* infections. In a recent report, a variant *Wolbachia* *w*MelPop-CLA has been introduced into the medically important mosquito disease vector *Aedes aegypti*. The infections induce mortality in older females which are responsible for majority of disease transmissions (McMeniman et al. 2009). Since a female mosquito must survive an extrinsic incubation period (EIP) to transmit dengue or other pathogens, a *Wolbachia*-induced shift in the population age structure toward younger females is expected to reduce pathogen transmission (Brownstin et al. 2003, Cook et al. 2008). In

addition, *w*MelPop-CLA infections have been demonstrated to inhibit the proliferation of several pathogens in the insect vector such as Dengue virus, Chikungunya virus and plasmodium within the mosquitoes (Moreira et al. 2009b). The combined effect of life shortening and pathogen inhibitory effects suggest the usefulness of replacing natural populations with individuals harboring the *Wolbachia* infection.

The *w*MelPop-CLA infection is also characterized as 'virulent' *Wolbachia*, as it imposes physiological costs and affects host fitness in various ways other than the shortened life span, which can potentially hinder the proposed strategy utilizing *Wolbachia*. The underlying mechanism of such effects were associated with unusual *Wolbachia* replication in host cells which leads to high density infections that consume resources in host cells, and eventually damage the host cells or tissues (Min and Benzer 1997, McMeniman et al. 2008). For example, the viability of infected eggs quickly declined over time resulting in reduced egg hatch (McMeniman and O'Neill 2010). Modeling studies indicated this decline in hatching success can significantly reduce the rate of population replacement by infected types (Turelli 2010, Yeap et al. 2011). The effects of the virulent *Wolbachia* infection have also been demonstrated to affect host behaviors. For example, *w*MelPop infections have been shown to reduce female biting ability as they age (Moreira et al. 2009a, Turley et al. 2009), or to be hyperactive with increased metabolic rate for entire adult life time (Evans et al. 2009). However, while a number of reports demonstrate impact of *w*MelPop infection on the fitness of various life stages of *A. aegypti*, there is relatively little information on the effect of *Wolbachia* on immature fitness and competitiveness as competition can dramatically affect mosquito population dynamics.

Immature competition is a key determinant in mosquito survival (Livdahl and Willey 1991, Juliano 1998, Juliano and Lounibos 2005). Subsequently the close examination of the relative competitiveness of *Wolbachia* infected larvae in relation with all possible competitors is vital to the success of *Wolbachia* invading a population. Using the example provided by the applied strategy underway in Australia, infected individuals released into the wild as a part of population replacement strategy will compete with resident uninfected larvae. The larval community is typically age structured in natural habitats, thus the competition involves various interactions between immature individuals of multiple larval developmental stages. Assuming infected eggs hatch in natural breeding sites where uninfected wild type populations dominate, the infected first instars must compete not only with similar aged larvae but also with older larger larvae.

In this study, I test the hypothesis of equal competitiveness between *Wolbachia* infected and uninfected first instars interacting with fourth instars of *A. aegypti* or *A. albopictus*. The experiments examine the relative survival of infected individuals at first instar stage during intra-specific competition with conspecific fourth instars, either *Wolbachia* infected or aposymbiotic. To evaluate inter-specific competition effects on the survival of the infected first instars, *A. albopictus* fourth instars are examined, which are frequently observed sharing natural habitats with *A. aegypti*. Furthermore, the rate of larval avoidance is quantified when responding to light stimulation to understand the underlying mechanisms of *Wolbachia* infection on host behaviors. The discussion links the immature interactions and the *Wolbachia* effects on host behaviors to understand *Wolbachia* infection dynamics in larval populations.

## Materials and Methods

**Insect strains.** Experiments used wild caught *A. albopictus* (Lexington, KY) (MID) naturally infected with two *Wolbachia* strains, *A. aegypti* (JCU) naturally uninfected (McMeniman et al. 2009), the *w*MelPop-CLA infected colony of *A. aegypti* (PGYP1), and *w*MelPop removed *A. aegypti* (PGYP1.tet) (McMeniman et al. 2009). Maintenance of *A. aegypti* mosquitoes was as previously described (McMeniman et al. 2009). In brief, all maintenance and experiments were conducted at 28 ± 2 ºC, 75 ± 10% RH, and a photoperiod of 18:6h (L:D). Eggs were submerged in a mixture of fish food (TetraMin Tropical Tablets, Tetra, Germany) in 400 ml of water. Larvae were given fish food *ad libitum* and adults were transferred into 30 × 30 × 30cm cages with constant access to a 10% sucrose solution. The females were blood fed with an artificial feeder using human blood collected at a blood bank (Kentucky Blood Center, Lexington, KY) or an anesthetized mouse (A3336-01; PHS Assurance). MID strain was maintained as previously described (Dobson et al. 2001).

**Immature competition assay.** Two experiments were conducted. In the first experiment, 30 1$^{st}$ instar (L1) of PGYP1 and PGYP1.tet strain (< 2 hours post hatch) were transferred into separate petridishes (60mm × 15mm) (BD BioSciences, Franklin Lakes, NJ) with 10ml fish food solution (0.1%). The experiment consisted of three treatments to test the effect of 4$^{th}$ instars (L4) of different mosquito strains on L1 survival. In the control treatment, no L4 were introduced. The other two treatments received six L4 (4 days old) of PGYP1 or PGYP1.tet. Surviving L1 were counted after 48 hours. Any pupated L4 were replaced with the cohort of L4 at 24 and 36 hours after the experiment was initiated.

The second experiment used same protocol as described above, but with different strains of mosquito larva. PGYP1 and JCU strains were used as L1, and JCU and MID strains were used as L4. Both experiments were replicated six times. Generalized linear models were used to examine for an effect of predator and/or prey type on the survival of L1 (binomial distribution with Logit link; JMP 8.0.1; SAS Institute, Cary, NC). Chi-square tests (two-tailed Fisher's exact) were used to compare the survival of L1 within treatments.

**Light response assay.** Two experiments were performed to estimate larval avoidance rate to light stimulation. In the first experiment, L4 of PGYP1, PGYP1.tet and JCU strains were tested using a rectangular container with a darkened area at one side, simulating a refuge (Fig. 5.1). The container was filled with 300ml water and a tube was placed within the center of the bright area in the container. Thirty L4 (4 days old) were reared in optimal conditions as described above and then transferred into the tube and allowed to acclimate in the dark condition for two minutes. The container was then exposed to a fluorescent light (Helical 20W; General Electric, Milwaukee, WI) and the tube was removed, releasing the larva. The larval movement was recorded using the CCD (charge-coupled device) camera with a resolution of 1280 × 720 pixels (iPhone 4; Apple, Cupertino, CA). In two-second intervals the video was paused and the number of larvae that reached the refuge was recorded until all larvae found the refuge. The experiment was replicated 20 times.

In the second experiment, L1 of PGYP1, PGYP1.tet and JCU strains were tested within the arena, following the similar protocol described above. Instead of rectangular container, a petridish (60 × 15mm) (BD BioSciences, Franklin Lakes, NJ) was used. The

petri dish contained 20ml water with an edge constructed of paper to simulate a refuge (Fig. 5.2). The petridish was placed on fluorescent light table (Porta-Trace Light table; Gagne, INC, Johnson City, NY), and 30 larvae were released from a tube positioned at a center of petridish after two minutes of acclimation time in the dark condition of tube. The larval movement was recorded using the DSLR (digital single-lens reflex) camera with a resolution of 1920 × 1080 pixels (SLT-A55VL; Sony Digital, Tokyo, Japan) mounted with a macro-photography lens (SAL30M28; Sony Digital, Tokyo, Japan) for 120 seconds. The video clip was analyzed as described above.

To compare the avoidance rate, the median time of larvae that reached the refuge was compared using a Kruskal-Wallis or one-way ANOVA test. To test for an effect of infection status on avoidance rate a Mann-Whitney test or student's *t*-test was used (JMP 8.0.1; SAS Institute, Cary, NC).

**Results**

**Immature competition assay.** In the first experiment, a significant effect of predator × prey type on L1 survival was observed ($\chi^2 = 9.09$, df = 2, $P < 0.05$) while no effect of predator or prey type was observed. Specifically, the survival of L1 was not different between PGYP1 (29.3 ± 0.3; mean number surviving ± SE) and PGYP1.tet strain (28.8 ± 0.4) with the absence of L4 by within-treatment test (Chi-square test; $P = 0.54$) (Fig. 5.3A). Similarly, survival was not different between PGYP1 (28.7 ± 0.2) and PGYP1.tet strain (28.7 ± 0.3) with the presence of L4 PGYP1 ($P = 1$). In contrast, a significant difference in L1 survival was observed when co-occurring with the uninfected L4 larvae.

Particularly, the survival of PGYP1 strain (26.8 ± 0.7) was significantly lower than PGYP1.tet (29.3 ± 0.3), with the presence of L4 PGYP1.tet ($P < 0.05$) (Fig. 5.3A).

A second experiment was conducted in which naturally-uninfected and artificially-infected L1 larvae were exposed to wild type *A. albopictus* (MID) and *A. aegypti* (JCU). The survival of L1 was not different between PGYP1 (29.2 ± 0.7) and JCU strain (29.7 ± 0.5) in the absence of L4 by within-treatment test (Chi-square test; $P = 0.45$) (Fig. 5.3B). However, the survival of L1 PGYP1 strain (24.8±0.8) was significantly lower than JCU (27.8 ± 0.5) in the presence of L4 JCU ($P < 0.01$). Similarly, the survival of L1 PGYP1 strain (24.5 ± 0.6) was lower than JCU (27.2±0.8) with the presence of L4 MID ($P < 0.05$). Significant effects of predator ($\chi^2 = 40.65$, df = 2, $P < 0.0001$) and prey ($\chi^2 = 8.57$, df = 1, $P < 0.01$) on the survival of L1 were observed while no effect of predator × prey interaction was observed ($\chi^2 = 0.21$, df = 2, $P = 0.9$).

**Light response assay.** To test for a hypothesized difference in larval escape response, an experiment was conducted in which *Wolbachia* infected and uninfected larvae were exposed to light and their response time in seeking a dark refuge was examined. A significant difference was observed in the median time of 4$^{th}$ instar reaching refuge in the comparison of three mosquito strains (Kruskal-Wallis test; $\chi^2 = 9.03$, df = 1, $P < 0.05$). PGYP1 was the slowest to find the refuge (12.4 ± 0.72 sec; mean seconds ± SE; n = 20), JCU intermediate (11.5 ± 0.6 sec; n = 20) and PGYP1.tet the fastest (10.4 ± 0.5 sec; n = 20) (Fig. 5.4A). The *Wolbachia* infected larvae (PGYP1 strain) were significantly slower to reach the refuge than the uninfected larvae (PGYP1.tet and JCU strains) (10.9 ± 0.5 sec) when compared by infection status of larvae (Mann-Whitney test; $\chi^2 = 6.772$, df = 1, $P < 0.01$).

A similar pattern was observed testing L1 in the second experiment. A significant difference was observed in the median proportion of larvae reaching refuge in the comparison of three strains. PGYP1 was slowest to find the refuge (16.3 ± 1.2 sec; n = 8), JCU intermediate (14.8 ± 0.9 sec; n= 8) and PGYP1.tet fastest (12.2 ± 1 sec n = 11) (one-way ANOVA; $F_{2,24} = 4.356$, $P < 0.05$) (Fig. 5.4.B). The infected larvae of PGYP1 strain were significantly slower to reach the refuge than the uninfected larvae of PGYP1.tet and JCU strains (13.3 ± 0.7 sec), when compared by infection status of larvae (student's *t*-test; df = 25, $P < 0.05$).

**Discussion**

Increased mortality of L1 was associated with presence of L4 and *Wolbachia* infection status of larvae. A physiological cost of *Wolbachia* on the host is attributable to the observed mortality of L1. A genomic study of *w*Mel strain of *Wolbachia* indicated the lack of complete metabolic pathways and limited ability to synthesize metabolic intermediates (Wu et al. 2004), suggesting genetically similar strain of *w*MelPop-CLA requires utilization of energy resource importing from hosts. The density of *w*MelPop-CLA or *w*MelPop was previously reported to be relatively higher in adult or embryonic stages comparing to other avirulent *Wolbachia* strains (Min and Benzer 1997, McGraw et al. 2002, McMeniman et al. 2008, Suh et al. 2009), which subsequently could be affecting the host by competing for host cell resources. It is consistent with the observed results in the larval stage that the phenotypic consequences of *w*MelPop-CLA infections in adults (McMeniman et al. 2009) and embryonic stages (McMeniman and O'Neill 2010) are virulent in *A. aegypti*.

L1 susceptibility to *w*MelPop-CLA virulence can increase due to larval resource competition and/or physical disturbance among larvae. Infected L1 can suffer food shortage by 1) competitive nutritional uptake by uninfected L4 and 2) physical contacts between L1 and L4, which can disturb feeding behavior of L1. Crowding (i.e., higher larval density) has been shown to be associated with increased mortality and development time, while resource was not a limiting factor (Wada 1965, Dye 1984). Feces from L4 could have been additional resources for L1, since L4 excreted feces during the experiments. However the results did not support the feces were utilized by infected L1.

Interestingly, dead L1 were not located after 48 hour of interactions between L1 and L4. As discussed above, the observed mortality of L1 could be a direct *Wolbachia* effect, and subsequently L4 larvae were consuming the carcass tissue. However, an alternate hypothesis is that the disappearance of L1 was the result of uninfected L4 consuming or preying on infected L1 larvae. L1 mortality can be explained by predation if it involved 'attacking behavior" of L4 and if it is defined as "an interaction in which one free-living individual kills and derives resources from another organism" (Abrams 2001). Cannibalistic or predatory behaviors have been often reported between larger and smaller larvae in *Aedes*, *Anopheles* and *Culex* species (Koenekoop and Livdahl 1986, Edgerly et al. 1999, Koenraadt and Takken 2003, Muturi et al. 2010). However, as most mosquito species feed on microorganisms and organismal detritus (Merritt et al. 1992), little has been studied on such behaviors in *A. aegypti* and *A. albopictus*. Since cannibalism was initially reported in *A. aegypti* (MacGregor 1915), Edgerly et al. (1999) reported that L4 of *A. aegypti*, *A. albopictus*, and *A. triseriatus* caused increased mortality in conspecific or interspecific L1. This suggests we cannot exclude possibility

that reduced survival of infected L1 is associated with predatory behaviors of *A. aegypti* and *A. albopictus* in this study. In order to confirm predation, the possibility of 'scavenging' on carcass should be excluded. Continuous visual observation to detect behaviors of L4 including attacking (i.e., chewing with mandibles) or killing L1 will provide evidence for predation but such visual evidence has been rarely provided in the previous studies. However, the results here are distinguished from the above studies in that the presence of L4 had larger impacts on the survival of infected L1 than the uninfected L1, suggesting that *Wolbachia* can potentially mediate or augment intra- or inter-specific predation within or between mosquito species.

To understand the underlying behavioral mechanism of the differential effects *Wolbachia* infection on host larvae, a larval response assay to light stimulation was performed. A sudden change in light condition was considered to be a predation risk. Larvae recognize visual change, thus they present avoidance behavior responding to dark subject approaching (potential predators) or brighter condition which stimulates them to find darker condition. The darker condition is considered as a refuge where predation risk is limited. Results suggest that *Wolbachia* infection affects larval behavior in both L1 and L4 by increasing the time spent seeking the refuge.

The possible mechanism for the delayed movement of infected larvae may be due to the *Wolbachia* infection increasing the metabolic rate of hosts inducing hunger and increases browsing behavior during avoidance process when larvae are exposed to potential predation risk. In a previous study, *w*MelPop-CLA was identified to induce increased metabolic rate throughout the life span of adult *A. aegypti* (Evans et al. 2009). Such effects could increase energy demand and hunger resulting in increased activity of

resource seeking behavior at larval stage. Similarly, a previous study reported that hunger increased predation risk of *A. triseriatus* to predator mosquitoes due to increased frequency of the browsing behavior, which predators utilize as cues to localize preys (Juliano et al. 1993). However, this cannot explain the result that infected L4 didn't affect L1 survival because higher mortality should have been observed in infected L1 (and/or uninfected L1) with the presence of infected L4, if the infected L4 were hungrier than the uninfected to increase browsing behavior searching for more resource including preys such as L1.

Second, the *Wolbachia* infection could directly alter larval behaviors. Wide distribution of *w*MelPop-CLA in *A. aegypti* includes ommatidia cells in eyes, brain neuronal cells or muscle tissues (Moreira et al. 2009b). Thus it is possible that *Wolbachia* disturbs normal signaling process in nervous systems and/or affecting muscle functioning. A mechanism of predation avoidance behavior of mosquito larvae was associated with waterborne chemical cues (Kesavaraju and Juliano 2010). Additional studies demonstrated that the prey limited their activity by responding to the cues to reduce predation risk (Kesavaraju and Juliano 2004, Kesavaraju et al. 2007). Thus, the infected larvae could be less sensitive to predation risks if the *Wolbachia* infection affected risk-sensing systems of larvae, resulting in increased susceptibility to predation. It is also possible that *Wolbachia* was causing malfunctioning of muscles to make larvae slow to avoid predation. For example, *w*MelPop-CLA was associated with modification of tissue characteristics known as 'bendy proboscis' which prevented infected females from normal blood feeding (Moreira et al. 2009a, Turley et al. 2009). These explanations are also consistent with the results that the infected L4 had no effects on both infected

and uninfected L1, suggesting the *w*MelPop infection prevented L4 from being effective predators to kill L1 or foragers to consume more resources affecting the survival of L1.

This study reveals a cost of the *Wolbachia* infection that can result in reduced competitiveness in infected L1 interacting with uninfected L4. Furthermore, infected L4 were determined to be poor competitors comparing to uninfected L4 because only uninfected L4 utilized infected L1 as food resource. Results suggest the presence of competing mosquitoes such as *A. albopictus* or other potential predators such as Toxorhynchites species that are often found in the natural habitats of *A. aegypti* could affect the population growth of *w*MelPop-CLA infected *A. aegypti*. Particularly, a wide distribution of *A. albopictus* has been observed in northern islands of Australia (Ritchie et al. 2006), suggesting a potential invasion of *A. albopictus* in the mainland of northern Australia. Possibly, *w*MelPop-CLA infected larvae will compete *A. albopictus* because the infected individuals are being released for a part of the population replacement strategy in the north Queensland of Australia (Iturbe-Ormaetxe et al. 2011) where *A. albopictus* were detected in proximity. In addition to *A. albopictus*, any potential intra- and inter- specific larval interactions will presumably impose fitness disadvantage of infected individuals, increasing the threshold that estimates the required number of *Wolbachia* infected mosquitoes needed in a population to enable *Wolbachia* spread and replacement (Turelli 2010). Current model studies that describe *Wolbachia* infection dynamics have not yet focused on the impacts of immature competition on *Wolbachia* spread. The observed effects of *Wolbachia* infections in immature mosquitoes presented here should be incorporated into population dynamic models, in order to better design population replacement strategies. Furthermore, the close ecological examination of

immature population dynamics of the target populations is required for the development of mosquito population replacement strategies.

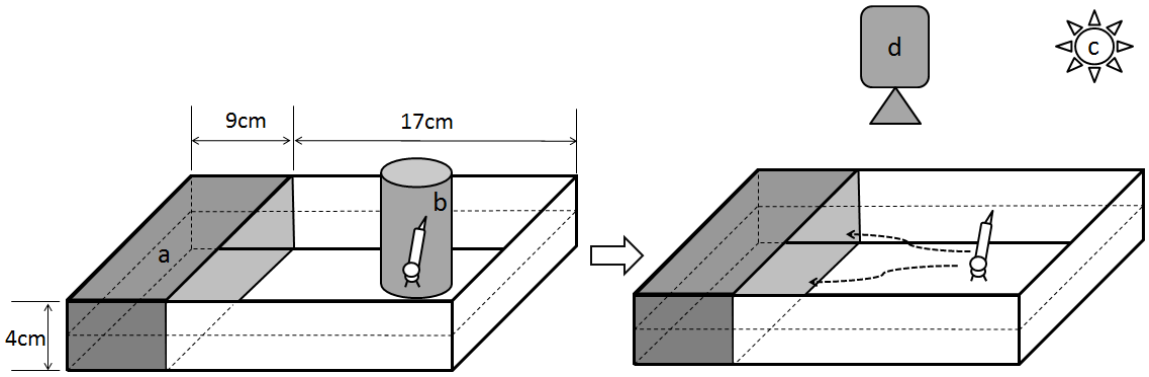


**Figure 5.1.** Schematic diagram of light response assay using 4th instar larvae (L4) of PGYP1, PGYP1.tet and JCU strains. A container with dark condition at one side was filled with 300ml water (a). A tube was used to acclimate 30 larvae in dark condition for two minutes (b). The larvae were released from the tube, and the larval avoidance rate to fluorescent light (c) was recorded using a camera (d).

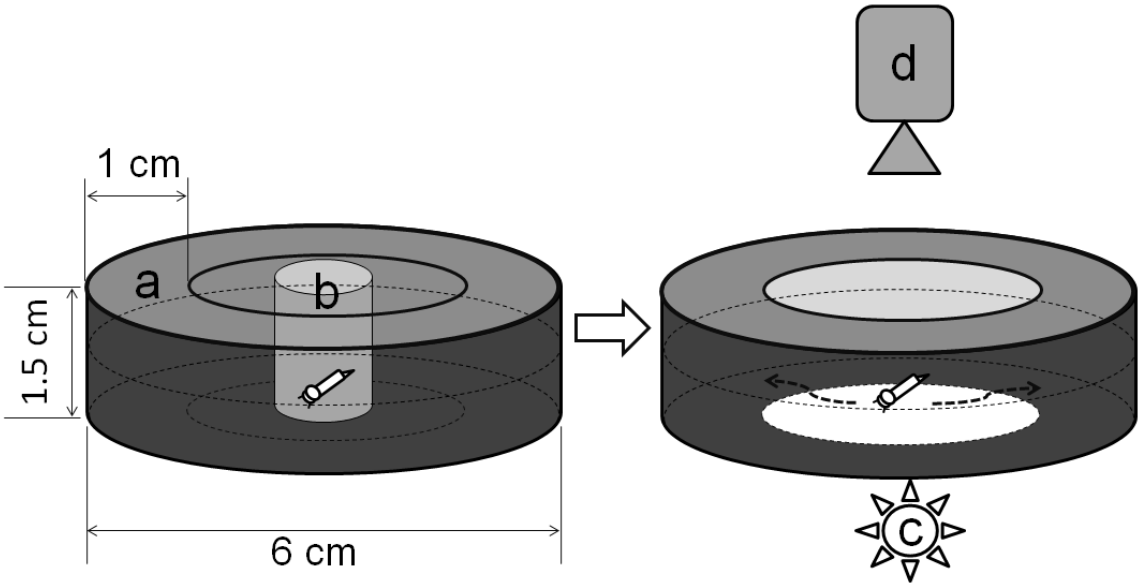


**Figure 5.2.** Schematic diagram of light response assay using 1st instar (L1) of PGYP1, PGYP1.tet and JCU strains. A petridish with dark condition around the edge was filled with 20ml water (a). A tube was used to acclimate 30 larvae in dark condition for two minutes (b). The larvae were released from the tube, and the larval avoidance rate to fluorescent light (c) was recorded using a camera (d).

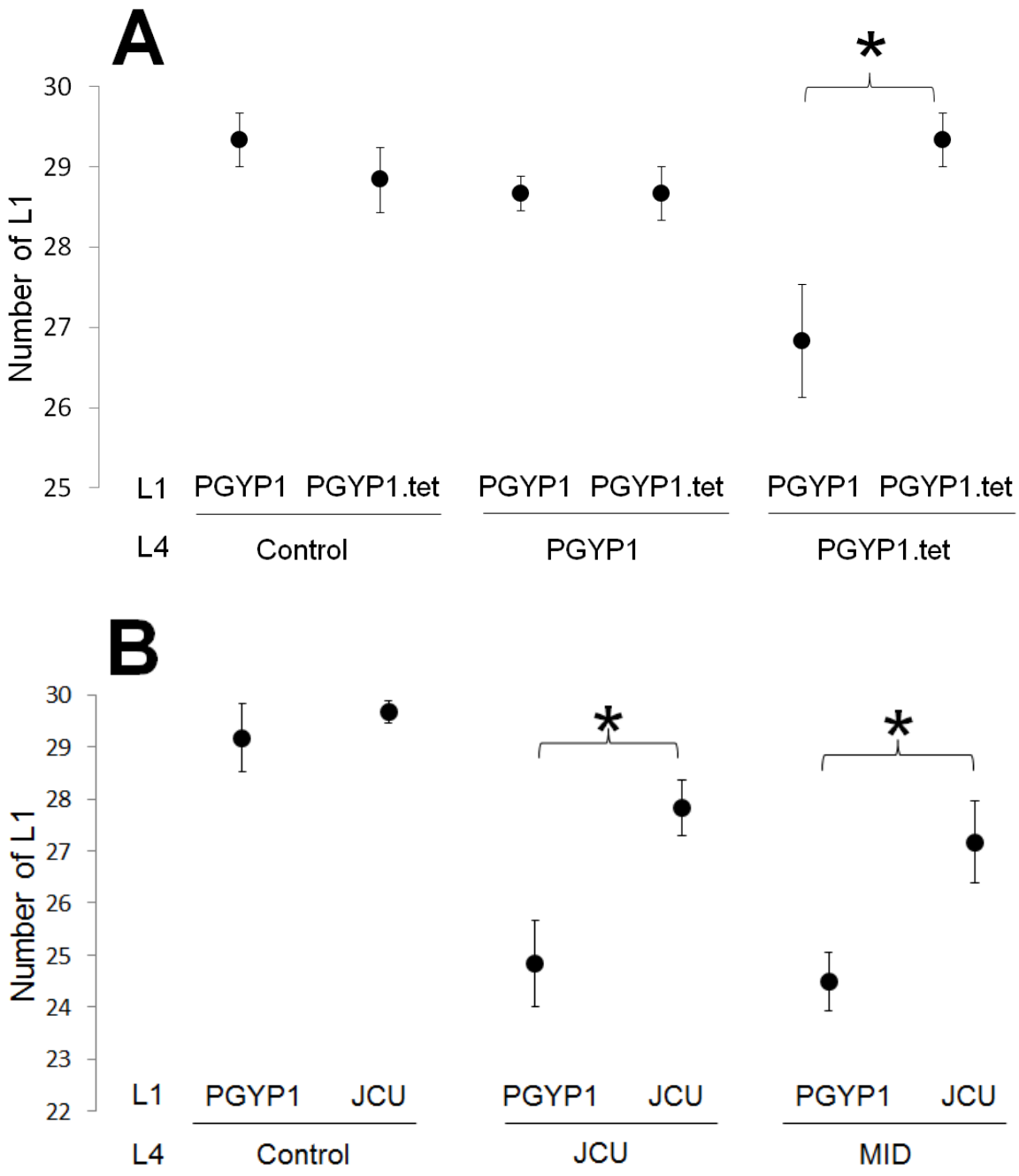


**Figure 5.3.** Survivorship of 1st instar from intra- (A) and inter- (B) specific interaction competing with six 4th instars. Asterisks represent significant difference at $P = 0.05$. Error bar = SE (n = 6).

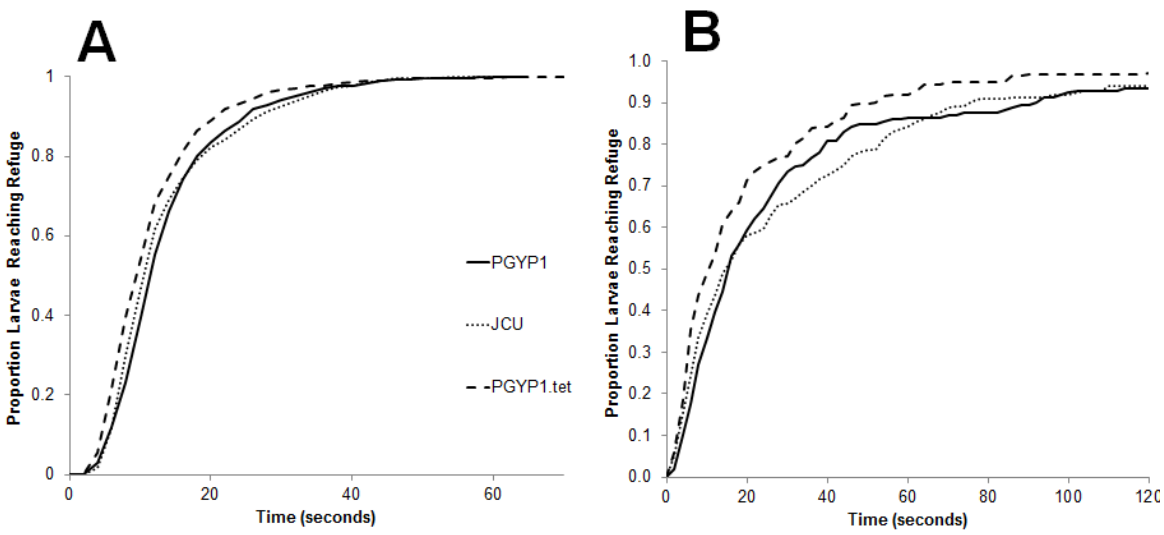


**Figure 5.4.** Larval avoidance rate to light stimulation estimated by proportion larvae reached refuge; 4th instar (A) and 1st instar (B).

# Chapter Six

## Comparative characterization of *Wolbachia* infections in *Aedes aegypti* transferred from *Aedes albopictus* and *Drosophila melanogaster*

### Introduction

*Wolbachia* are maternally inherited, intracellular bacteria, which occur within a wide range of arthropod species and nematodes (Werren et al. 2008). *Wolbachia* are responsible for a variety of reproductive alterations in infected hosts including parthenogenesis, feminization, male killing and cytoplasmic incompatibility (CI) (Werren 1997). CI is typically observed in females that are mated with males infected with a different *Wolbachia* type. While the mechanism of CI has not been defined, multiple models have been proposed, including the "modification/rescue" model (Werren 1997, Charlat et al. 2001). In this model, the sperm of infected males are "modified" by *Wolbachia*, resulting in karyogamic failure of fertilized eggs and early embryonic death. In embryos that carry the same *Wolbachia* strain as the male mates, the modified sperm are rescued, allowing for normal development.

Unidirectional CI is typically observed in the mating between an uninfected female and infected male. The resulting cost of CI to uninfected females in the population (i.e., reduced progeny from incompatible matings) provides a reproductive advantage relatively to infected females, which promotes the spread of *Wolbachia* into an uninfected population. CI can also be bidirectional, and occurs when mating partners have a different *Wolbachia* infection type.

The ability of *Wolbachia* to spread into populations has led to the suggested applied use of *Wolbachia* to spread desired genes (e.g., genes causing refractoriness to disease transmission). Specifically, infected individuals can be "seeded" into a population, and CI would drive the *Wolbachia* infection, replacing the uninfected cytotype. Both the threshold infection frequency required to initiate a replacement event and the subsequent rate of *Wolbachia* spread into a population are determined by the CI strength (i.e., the level of embryonic lethality), the maternal inheritance rate and the effect of *Wolbachia* on host fitness (Hoffmann et al. 1990). Replacement strategies have recently been proposed to use the *Wolbachia* that are associated the effects to potentially decrease vectorial capacity and/or competency of medically important *Aedes aegypti* populations (Xi et al. 2005a, McMeniman et al. 2009). Such strategies include the use of *Wolbachia* for gene drive to modify the age structure of populations. To accomplish this, different *Wolbachia* infections have been introduced into wild type laboratory colonies, which are naturally uninfected. Introduced *Wolbachia* infections include the *w*MelPop infection from *Drosophila melanogaster* (Min and Benzer 1997, McMeniman et al. 2009) and the *w*AlbB infection from *A. albopictus* (Xi et al. 2005a). The recent demonstration of *w*MelPop and *w*AlbB infections to reduce replication of human pathogens such as Dengue, Chikungunya, and Plasmodium (Moreira et al. 2009b, Bian et al. 2010) has suggested an additional use of transfected strains of *A. aegypti* in the population replacement strategies.

An additional *Wolbachia* based strategy known as the incompatible insect technique (IIT) involves inundative releases of *Wolbachia* infected males, which is analogous to sterile insect technique (SIT). SIT requires generation of sterile males,

which often causes loss of fitness of release males due to technologies applied (e.g., irradiation and chemosterilization) (Alphey 2002, Benedict and Robinson 2003). Similarly, *Wolbachia* based IIT involves generation of incompatible males, thus the effects of *Wolbachia* on host fitness are important. Specifically, released males compete with wild type males, and vector females that have mated with the incompatible males are sterilized resulting in no progeny, causing population suppression and/or elimination. Thus, no cost of infection on male mating competitiveness and a high level of CI can increase efficacy and efficiency of the strategy. In addition, no cost of *Wolbachia* infections on host reproduction (e.g., fecundity or egg hatch) will increase mass rearing efficiency.

Examples of *Wolbachia*-associated fitness costs and benefits have been reported, including a moderate to extreme physiological cost (Hoffmann et al. 1990, Fleury et al. 2000, Perrot-Minnot et al. 2002, Huigens et al. 2004, Rigaud and Moreau 2004, Duron et al. 2006, Islam and Dobson 2006, Suh et al. 2009, McMeniman and O'Neill 2010), an absence of cost (Harcombe and Hoffmann 2004, Montenegro et al. 2006) and benefits (Bandi et al. 1999, Hoerauf et al. 1999, Vavre et al. 1999, Dedeine et al. 2001, Dobson et al. 2002b, Fry et al. 2004, Hedges et al. 2008, Teixeira et al. 2008, Brownlie et al. 2009) in either naturally or artificially *Wolbachia* infected hosts. Prior studies have focused on characterizing an effect of *Wolbachia* infection on hosts by comparing uninfected aposymbiotic hosts, yet direct comparisons on different *Wolbachia* infections in a same host species is rare. A *Wolbachia*-associated fitness cost is predicted to decrease success of the population replacement strategy due to lowered competitiveness of infected hosts.

Thus, evaluating relative fitness costs of *Wolbachia* infections on a host will provide insights to better design the proposed strategies utilizing *Wolbachia*.

Here, I compare and characterize the two artificially *Wolbachia* infected mosquito strains. In this study, I examine the relative fitness of *w*MelPop and *w*AlbB infected *A. aegypti* by measuring longevity, fecundity, level of CI and male mating competitiveness. The results are discussed in reference to the potential for these artificially infected strains to be used as a potential population replacement or IIT strategy.

## Materials and Methods

**Insect strains.** The WB1 strain is *Aedes aegypti* that has been artificially infected with *w*AlbB, introduced by embryo microinjection with cytoplasm from *A. albopictus* into naturally uninfected *A. aegypti* (WACO) (Xi et al. 2005b). PGYP1 is *A. aegypti* artificially infected with *w*MelPop-CLA (McMeniman et al. 2009). PGYP1.tet is an uninfected *A. aegypti* strain resulting from tetracycline treatment of PGYP1. Mosquito strains were maintained as previously described (McMeniman et al. 2009). Mosquito colony maintenance and all experiments were at 28 ± 2 ºC, 75 ± 10% RH, and a photoperiod of 18:6h (L:D). Larval rearing conditions were optimal (i.e., low larval density and food provided *ad libitum*).

**PCR amplification.** DNA was extracted from adult mosquitoes as described previously (Brelsfoard et al. 2008). PCR amplification was performed in 25µl reaction volumes using Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) with 16s *Wolbachia* specific (Moreira et al. 2009b) and CO1 universal primers (Turley et al. 2009) to determine *Wolbachia* infection status and verify template quality, simultaneously;

17.5µl of $H_2O$, 2.5ul of 10X buffer, 0.8µl of dNTP (10mM), 0.5µl of W-specf, W-specr, CO1f and CO1r each (10µM), 0.2µl Taq and 2µl of DNA template. A MJ Research PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) was used to perform 94°C, 2 min and 38 cycles of 94°C, 2 min; 55°C, 45 sec and 72°C, 1.5 min followed by 72°C,10 min.

**Fecundity, CI and female longevity.** For the fecundity and CI assays, four crosses were conducted between PGYP1 and WB1 individuals (10 females × 10 males/cage; < 2 days old; n = 5). Adults were provided with a constant supply of 10% sucrose solution. For blood feeding, human blood (Blood center, Lexington, KY) was provided using Hemotek artificial blood feeding system (Discovery Workshops, Accrington, UK). The number of engorged and surviving females was counted after blood feeding. An oviposition cup lined with wet paper was continuously available to collect eggs from each gonotrophic cycle. Eggs were matured for three days and hatched to estimate fecundity and egg hatch. The number of eggs and arcsine transformed egg hatch were compared using repeated-measures analysis of variance (ANOVA) test with pair-wise comparisons to assess effect of cross type on fecundity with a Bonferroni adjustment at $P = 0.05$ (SPSS; SAS Institute, Cary, NC). Post-hoc Tukey honestly significant difference (HSD) tests were used to examine for a difference in fecundity among gonotrophic cycle. Kaplan Meir Log rank test was used to compare female longevity (JMP 8.0.1; SAS Institute, Cary, NC).

**Male age and CI.** WB1 and PGYP1 males were isolated as pupae and held virgin for different lengths of time (1, 10, 30 days) and then placed with PGYP1, WACO or WB1 females (< 2 days old) in cages for one week. Cages included a constant supply of 10% sucrose solution (15 females × 15 males). Human blood was provided for PGYP1, and

an anesthetized mouse (A3336-01; PHS Assurance) was provided for WACO and WB1 for blood feeding. Fully engorged females were transferred into individual oviposition cups to collect eggs. Eggs were hatched after 3 days of maturation to estimate egg hatch rate.

**Male mating competitiveness and longevity.** To test for a hypothesized equal competitiveness of mating ability between PGYP1 and WB1, 20 PGYP1 females (< two days old) were mated with differing ratios of PGYP1 and WB1 males (40:0, 30:10, 20:20, 10:30 and 0:40; < 2 days old) in a cage for seven days. Human blood was provided, and engorged females were transferred into individual oviposition cups to collect eggs. Mating competitiveness was estimated by comparing observed and predicted egg hatch per ratio. Observed egg hatch was calculated by combining hatched and unhatched eggs from all individual females within a cage. Predicted egg hatch (*P*) was calculated per cage using following equation as previously described (Fried 1970);

$$P = \frac{C(E_c) + I(E_i)}{C + I}$$

Where $C$ = number of compatible males (i.e., PGYP1 males), $I$ = number of incompatible males (i.e., WB1 males), $E_c$ = egg hatch in compatible cross (i.e., PGYP1 × PGYP1) and $E_i$ = egg hatch in incompatible cross (i.e., PGYP1 × WB1). Observed and predicted egg hatches were then compared using Chi-square test (two-tailed Fisher's Exact; JMP 8.0.1; SAS Institute, Cary, NC) in the WB1 male ratios of 0.25, 0.5 and 0.75. To assess potential effect of multiple mating, arcsine transformed egg hatch of hatching broods (i.e., compatible cross) from differing ratios of incompatible males were compared using Kruskal Wallis test (JMP 8.0.1; SAS Institute, Cary, NC). Daily

mortality of males was monitored for PGYP1 and WB1 males and log rank test was used to compare male longevity (JMP 8.0.1; SAS Institute, Cary, NC).

**Life table analysis.** A life table was constructed from the five gonotrophic cycles in the population of PGYP1 and WB1 as previously described (Reisen et al. 1984, Mahmood et al. 2004). Female age (x) and survivorship ($l_x$) was estimated by calculating mean values of age and survival rate at each gonotrophic cycle. Age specific fecundity ($m_x$) was calculated from egg number per female multiplied by egg hatch rate and sex ratio ( = 0.5; assumed as 1:1) representing female eggs per living female per gonotrophic cycle. Generation time (T) was estimated using age-specific reproductive effort ($l_x m_x$) and summed to estimate reproductive effort per generation ($R_0$). The approximate rate of population increase per female per gonotrophic cycle was calculated as $r_m = \ln(R_0)/T$. Doubling time ($D_t$) was calculated as $D_t = \ln(2)/r_m$. Parameters were compared (n = 5) using *t*-test between PGYP1 and WB1 populations (JMP 8.0.1; SAS Institute, Cary, NC).

## Results

**Fecundity.** Fecundity of PGYP1 females was lower than WB1 females only in the first gonotrophic cycle regardless of male partner (One-way ANOVA; $P < 0.05$). However, fecundity of PGYP1 and WB1 females was not significantly different (pair-wise comparison, Bonferroni adjusted; $P > 0.1$) if fecundity was compared for three gonotrophic cycles (Fig.6.1A).

**Bidirectional CI.** A pattern consistent with bidirectional CI was observed in crosses between PGYP1 and WB1 (Fig. 6.1B). No larva hatched among the 3064 eggs resulting from PGYP1 females mated with WB1 males (n = 5 cage replicates). Similarly, strong

CI was also observed in the reciprocal cross (i.e., WB1 females mated with PGYP1 males), with 25 larvae hatching from 3623 eggs (1.0 ± 1.8% Hatch; mean ± SD; n = 5) during the three gonotrophic cycles (Fig. 6.1B). In an additional experiment to assure CI ability of *w*MelPop and *w*AlbB in uninfected eggs, PGYP1.tet and WACO females were mated with PGYP1 and WB1 males in a cage (10 females ×10 males) and no hatching larvae were observed from 2326 eggs collected from 20 uninfected females for three gonotrophic cycles. Mean egg hatches during three gonotrophic cycles from PGYP1 and WB1 females mated with similarly-infected males were 51.9 ± 7.9% (mean ± SE; n = 15), and 53.0% ± 7.6% (n = 15), respectively and were not significantly different each other ($F_{1,28}$ = 0.016, $P$ = 0.901).

**Male age and CI.** Crosses between PGYP1 females and WB1 males up to 30 days old resulted in no hatch from 1754 eggs (Table 6.1). Similarly, no hatching larva was observed among 3417 eggs from WACO females mated with WB1 males. Egg hatch rates observed from cross between WB1 individuals (53.6 ± 6.2%; mean ± SE; n = 3) did not differ from egg hatches observed in the previous mating experiment observed during three gonotrophic cycles (53 ± 5.3%; n = 3) (Fig. 6.1B).

**Male mating competitiveness.** Egg hatch resulting from crosses of PGYP1 females and PGYP1 or WB1 males was not significantly different for all three ratios of incompatible male cages ($P$ > 0.5) (Fig. 6.2). Observed egg hatch was the highest in the compatible male cage (no WB1 males), and egg hatch decreased as the ratio of incompatible males increased. When PGYP1 females mated with WB1 males, no single larva was hatched from 3745 eggs. No significant difference was observed in egg hatch from the

incompatible crosses among four different ratios of incompatible male cages (i.e., egg hatch > 0) (Kruskal - Wallis; $\chi^2 = 1.4$, df = 3, $P = 0.281$).

**Adult longevity.** Longevity of PGYP1 was significantly reduced compared to WB1 in both females and males (Fig. 6.3). Median longevity of PGYP1 and WB1 females were 44 days and 36 days, (Log rank; $\chi^2 = 34.13$, df = 1, $P < 0.0001$), males were 18 days and 19 days (Log rank; $\chi^2 = 6.60$, df = 1, $P = 0.010$), respectively.

**Life table analysis.** Life table analysis showed no difference in net reproductive rate ($R_0$), and intrinsic rate of increase ($r_m$), and doubling time ($D_t$) between PGYP1 and WB1 population (Table 6.2). However, generation time (T) was significantly longer in WB1 population (19.2 ± 0.62 days; mean ± SE) comparing to PGYP1 (17.1 ± 0.63) ($F_{1,8} = 5.61$, $P = 0.045$).

## Discussion

*Wolbachia*-based strategies require assessment of *Wolbachia* effects on host fitness, since negative fitness effects could potentially reduce replacement efficiency and reduce the competitiveness of released males as part of an IIT strategy. Examination of *Wolbachia* effects on host phenotypes typically involves the introduction of novel *Wolbachia* infection types and the generation of aposymbiotic uninfected hosts. Previous studies have used this method for *A. aegypti* adults to evaluate fitness costs of *w*MelPop-CLA infections, and the results suggested significant reduction in adult longevity but no cost on fecundity (McMeniman et al. 2009). Superinfection that includes *w*AlbA and *w*AlbB were described previously (Dobson et al. 2004, Islam and Dobson 2006, Gavotte et al. 2009). However, the previous studies did not exclude an interaction effect of

*w*AlbA infection, and little information is available for *w*AlbB infection regarding fitness cost. Here, I directly compared two potential agents for the proposed strategy; *w*MelPop-CLA infected (PGYP1) and *w*AlbB infected *A. aegypti* (WB1) by evaluating relative effects of *Wolbachia* infection on host fitness.

A significant difference in fecundity was observed in the first gonotrophic cycle between PGYP1 and WB1 strain, although overall fecundity didn't differ among cross type during first three gonotrophic cycles (Fig. 6.1). No fecundity cost in the PGYP1 strain was reported in the prior study looking at the first gonotrophic cycle (McMeniman et al. 2009). The observed increase in fecundity of WB1 when compared to PGYP1 at the first gonotrophic cycle could be explained by a positive effect of the *w*AlbB infection in *A. aegypti*. A direct comparison between WB1 and naturally uninfected individuals will be required to test the hypothesis.

Survivorship of PGYP1 was reduced when compared to WB1 in both females and males similar to the prior study (McMeniman et al. 2009), while longevity of WB1 females were not significantly different from uninfected individuals (Bian et al. 2010). Although the shortened lifespan of females is expected to reduce disease transmission by old females, it can simultaneously affect net reproduction of infected hosts and relative competitiveness by increasing number of seeding individuals to reach unstable equilibrium threshold (Turelli 2010). In reference to an IIT strategy, the increased longevity of WB1 males may increase the chance of WB1 males mating with the uninfected females. However, studies comparing WB1 and naturally uninfected males will need to be conducted.

Parameters in the life table analysis using data from fecundity, egg hatch and survivorship of PGYP1 and WB1 showed no difference in reproduction between the two strains. The only difference was observed in generation time, presumably due to significantly reduced female longevity by *w*MelPop-CLA infection. The shortened life span of PGYP1 did not result in a decrease in population growth rate, possibly because most eggs are produced by younger females before the reduced survival is expressed. This will allow PGYP1 females to avoid fitness cost at younger age and produce offspring equally productive as wild type or WB1 females. However, as discussed in a prior study, life-shortening effects can influence invasion power depending on age structure (e.g., overlapping generation) in more field like model system (Turelli 2010).

*w*MelPop-CLA and *w*AlbB expressed strong bidirectional CI in *A. aegypti* but the pattern of CI was different from the previous studies. The CI induction by PGYP1 males in WB1 eggs allowed survival of 25 larvae, which was not consistent with the previous study showing 0% egg hatch in unidirectional CI crosses (McMeniman et al. 2009). The loss of CI can be explained by an unexpected partial rescue effect of *w*AlbB over *w*MelPop-CLA modification. Similarly, *w*AlbB infections showed strong bidirectional CI with *w*AlbA in *A. albopictus* but the egg hatch was not 100% and 2~4 % of eggs survived to larvae (Xi et al. 2005b). However, *w*AlbB induced perfect CI in *A. aegypti* showing 0% egg hatch in bidirectional CI (Fig. 6.1B) as well as unidirectional crosses (table 6.1). The results suggest that the pattern of bidirectional CI can be affected by both host species and *Wolbachia* strains.

No significant difference was observed between predicted and observed egg hatches in the male mating competition assay, suggesting that WB1 males are equally

competitive as PGYP1. If *Wolbachia* infection affected the mating ability of the host, *w*MelPop-CLA or *w*AlbB may equally decrease (or increase) the male competitiveness. However, it is more likely that there is no effect of *Wolbachia* infection on male mating ability since no difference in male mating competitiveness was observed in the comparison of *w*MelPop-CLA infected and uninfected males (see chapter four). Results from the present study also suggest that females mated and used sperm to fertilize her eggs from only one male. This is consistent with prior studies in *A. aegypti* that this species is in most cases monogamous (Clements 1999). In addition to the constant level of CI regardless of male age, WB1 males are expected to effectively cause CI in uninfected females through their life-time during the invasion of infected hosts.

Observed results suggest that both PGYP1 and WB1 could be used as a *Wolbachia* based replacement due to their refractoriness to diseases (Moreira et al. 2009b, Bian et al. 2010), high rates of CI, and minor effects on host fitness. However, a number of recent reports support the fitness cost of *w*MelPop-CLA infections by reduced longevity, fecundity and egg viability (McMeniman and O'Neill 2010, Yeap et al. 2011). Here, my results strongly support that WB1 can be additional or alternative agent for replacement study with no cost on egg viability (Xi et al. 2005a), although direct comparison with naturally uninfected *A. aegypti* in the target area looking at life history traits will provide specific information for the release study. In addition, complete bidirectional CI observed in this study also would allow a strategy of using *w*AlbB to suppress a population that has been previously infected with the *w*MelPop infection.

**Table 6.1.** Effect of male age on CI; percent egg hatch ± SE and (number of replicate crosses)

| Cross (female × male) | Percent egg hatch for male age of | | |
|---|---|---|---|
| | 1 day | 10 days | 30 days |
| PGYP1 × WB1 | 0.0 ± 0.0 (16) | 0.0 ± 0.0 (14) | 0.0 ± 0.0 (13) |
| WACO × WB1 | 0.0 ± 0.0 (14) | 0.0 ± 0.0 (11) | 0.0 ± 0.0 (14) |
| WB1 × WB1 | 65.7 ± 6.4 (15) | 52.6 ± 10.8 (11) | 43.5 ±11.4 (12) |

**Table 6.2.** Life table data of PGYP1 and WB1 strains (mean ± SE)

| Strain | $R_0$ | T | $r_m$ | $D_t$ |
|---|---|---|---|---|
| PGYP1 | 15.93 ± 1.59 | 17.07 ± 0.63[b] | 0.16 ± 0.01 | 4.33 ± 0.24 |
| WB1 | 25.49 ± 4.75 | 19.17 ± 0.62[a] | 0.17 ± 0.01 | 4.28 ± 0.31 |

Different letters represent significant difference at $P = 0.05$.

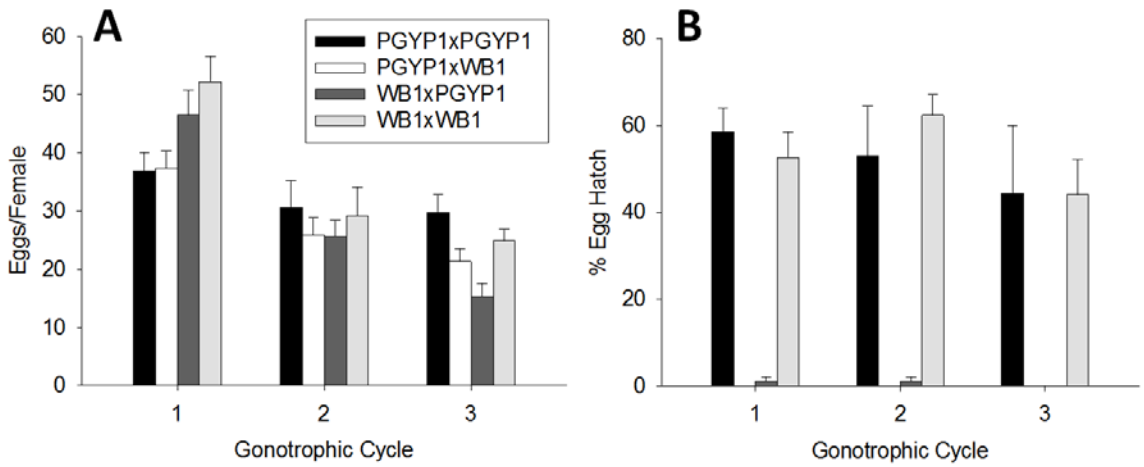


**Figure 6.1.** Fecundity (A) and egg hatch (B) resulting from PGYP1 and WB1 crosses during three gonotrophic cycles. Error bar = SE (n = 5).

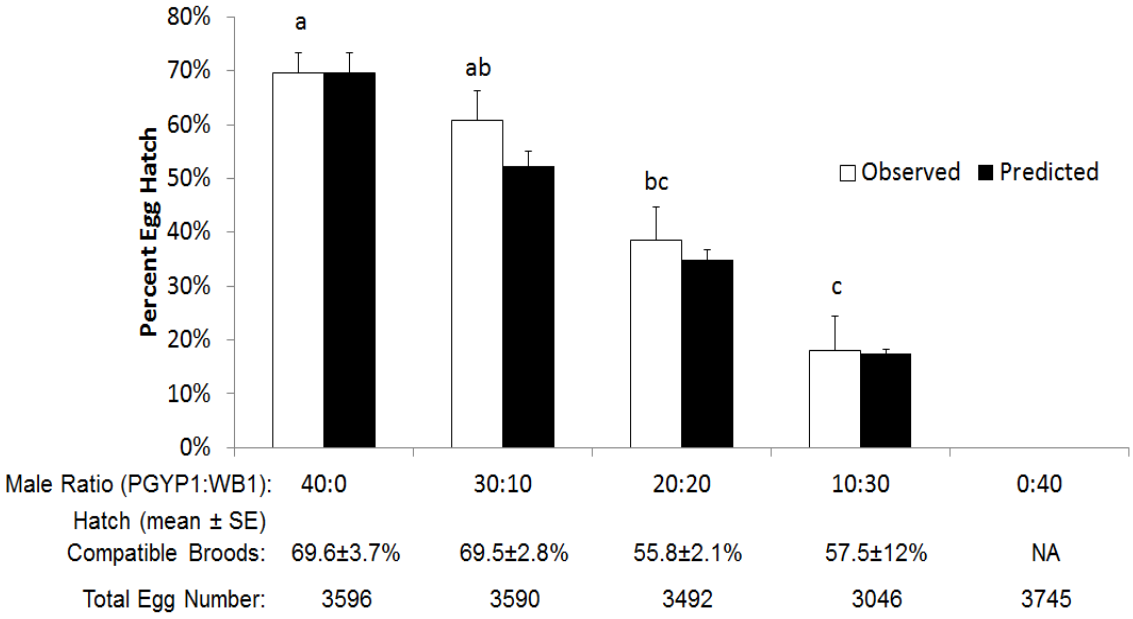


**Figure 6.2.** Male mating competitiveness between PGYP1 and WB1 males compared by observed and predicted egg hatches resulting from PGYP1 females mated with differing ratios of PGYP1 and WB1 males (error bar = SE; n = 5). Observed egg hatch was calculated as egg hatch per cage. Predicted egg hatches were calculated assuming equal competitiveness of PGYP1 and WB1 males.

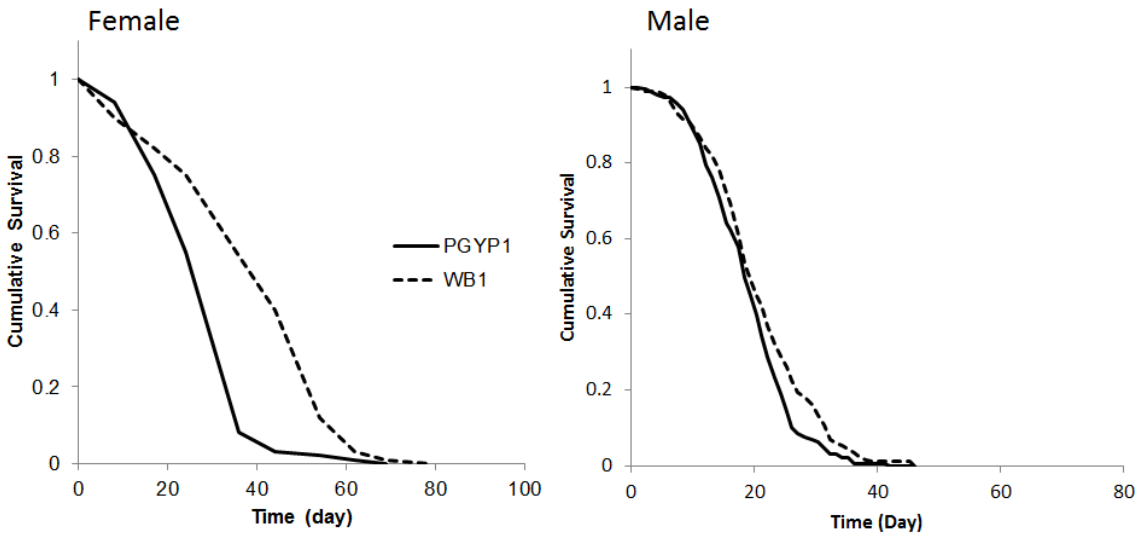


**Figure 6.3.** Female and male longevity of *A. aegypti* infected with *w*MelPop-CLA (PGYP1, solid line) or *w*AlbB (WB1, dashed line).

## Chapter Seven

## Immature mosquito screening system

### Introduction

Living modified organisms (LMO) have been a growing interest, as they are expected to aid in vector controls. One idea is to replace wild population with LMO that can exhibit lowered vectorial capacity and/or competency in the field. This method usually requires utilizing selfish elements, which can confer carriers with potentials to spread into natural vector population. Examples include an endosymbiont *Wolbachia* (Werren et al. 2008) or genetically modified organisms (Chen et al. 2007, Windbichler et al. 2011), which have been suggested as gene driving vehicles to deliver desirable traits such as disease refractoriness. Another is to release LMO that are capable of inducing sterility in wild populations, which can result in suppression and/or elimination of vectors. Such LMO have been also generated via introduction of *Wolbachia* infection (Brelsfoard et al. 2008) or genetic modification (Alphey 2002). In addition, studies on vector controls often require introduction and maintenance of vector insects and/or infectious agent, hence accidental release of these insects from confined areas can be problematic. Vectors that carry infectious diseases can be a direct threat to workers in the laboratory and public health of a local community. Pathogen free vectors also have potential to increase or establish disease transmission as examples are found in introductions of exotic species mostly resulting in increased risk of pathogen transmission (Benedict et al. 2003). Moreover, accidental release of the carriers with selfish elements can cause unwanted impacts on the environment or failure of the proposed strategies.

Arthropod containment guidelines have developed useful protocols of containment methods (Benedict et al. 2003). Specifically, arthropods or vectors should be contained within multiple barriers (e.g., screened cages or screened room) to minimize risk of escape. Safety manuals were developed for each containment level where risk level is elevated as contained vectors or infectious agents are classified to be more dangerous (Scott 2005). Discharging of wastewater is suggested to disinfect eggs or immature individuals at aquatic stage by either using traps or treatments such as heating or freezing (Scott 2005). However, no specific tools or methods have been further suggested to facilitate the discarding process. Particularly when mass rearing of mosquitoes is necessary, freezing or heating of wastewater can become increasingly difficult due to its amount of wastewater produced. Thus, development of traps that are capable of filtering large amount water and immature individuals is required for sustained prevention of vector escapes.

Here, a model system is described to filter immature mosquitoes from wastewater. Results suggest laboratories that rear mosquitoes can use this technique to reduce the potential for unwanted release of laboratory mosquitoes as well as facilitate mass rearing programs. The described system uses commercial sieves of several pore diameters to filter immature mosquitoes from wastewater.

## Materials and Methods

**Insect strains.** To determine applicability to multiple species, the system was tested with three wild type aedine species. Mosquitoes used in experiments were the APM strain of *Aedes polynsiensis* (Dean and Dobson 2004), the MID strain of *A. albopictus* (Lexington,

KY), and the WACO strain of *A. aegypti* (Mains et al. 2008). Mosquitoes were maintained as described previously (Dobson and Rattanadechakul 2001).

**Immature mosquito and filtering system.** A total of eight age groups of immature mosquitoes were tested to represent different ages or stages of immature individuals. These groups included: eggs and larvae at age of 2, 24, 48, 72, 96, 120, 144 hours. The hatching larvae were provided with liver powder solution *ad libitum*.

Based on a preliminary test, a series of USA standard sieves (Hogentogler & Co. Inc., Columbia, MD) with differing opening sizes were selected that can collect immature individuals with various ages including eggs and 1$^{st}$ instars (Table 7.1). Seven sieves of different opening sizes were stacked in the order of opening size with the smallest (sieve no. 120) at the bottom and the largest (sieve no. 10) at the top. Approximately 50 individuals from each immature group were poured on the top sieve and tap water was used to rinse the sieves for 1 min (~2 liter), and the number of individuals collected in each sieve was counted. The experiment was repeated five times per mosquito strain.

**Immature size estimation and statistical analysis.** The mean number of immature individuals collected in each sieve was calculated to estimate proportional distribution of filtered individuals in the stack of sieves for the all three mosquito species combined. The mean filtering opening size of each immature group was estimated by calculating the average opening size of sieves where immature individuals were collected (i.e., sieve opening size = size of collected immature individuals).

The mean filtering opening size were assumed as the size of each age group and compared between strains or among sieve sizes to determine strain specific size variance within a same age group or age specific size variance. ANOVA tests were used with ad

hoc Tukey honestly significant difference (HSD) for any mean comparison analysis and proportion data were arcsine transformed to meet normality (JMP 8.0.1; SAS Institute; Cary, NC).

**Results and Discussion**

Here, I present a methodology to separate eggs and immature mosquitoes from wastewater to minimize the risk of accidental release of biological material. All immature mosquitoes (total 4661) of three aedine species were successfully collected with sieves used in the experiment. While different sieves collected different sizes of eggs and immature mosquitoes, the smallest sieve size (no. 100 with an opening size of 0.149 mm) allowed no eggs or immature mosquitoes to pass through (Fig. 7.1). No eggs or immature individuals were detected in water collected in a container that has passed through all test sieves. Here, I suggest to use sieve no. 100 as a final filter to collect immature individuals in order to increase filtering efficiency, because finer sieves (e.g., sieve no.120 or greater) are more likely to be clogged by collecting smaller particles besides eggs or $1^{st}$ instars.

Previous studies have shown that egg size can be dependent on female size of *Aedes aegypti* (Schofield and White 1984, Steinwascher 1984). However, another study describes egg size was not correlated with female size in *Aedes cantans* (Renshaw et al. 1994). These studies suggest that egg size variance can be interspecific and dependent on larval density that determines female size. However, egg size was not significantly different among species in our study and the mean egg size of three species was estimated as 0.210 ± 0.0087mm (mean ± SD; n = 15) (Fig. 7.1), indicating most of eggs

were collected in sieve no. 60 (opening size = 0.250mm) and no. 80 (0.177mm). Since the proportion of individuals observed in sieve no.100 was less than 1%, we suggest sieve no.100 is most likely to collect smallest immature mosquitoes.

The proportion of immature mosquitoes collected was significantly different among sieves (ANOVA with Tukey HSD; $F_{4,70} = 351.6$, $P < 0.0001$). Forty-nine percent of all immature individuals (including eggs) of three mosquito species were collected at sieve no. 20, 19% at sieve no. 40, 19% at sieve no. 60, 12% at sieve no. 80, and 1% at sieve no. 100 (Fig. 7.1). Use of one particular sieve may cause clogging due to accumulated immature individuals, thus the filtering proportion of sieve 20 needs to be distributed. For example, using more sieves between no. 10 and 40 can reduce filtering proportion of sieve no. 20 below 49%. Potentially, using multiple sieves instead of one smallest sieve will help avoid clogging and subsequently increase filtering efficiency when filtering large quantities of wastewater.

Size estimates of immature mosquitoes using the sieve opening size showed species-specific size variance within a same age group (Fig. 7.2). WACO was larger at age 0, 48 and 72 hours comparing to MID or APM (ANOVA with Tukey HSD; $P < 0.05$), suggesting that WACO has either a faster developmental rate or is genetically predisposed to be larger, thus filtering distribution of sieves can be different depending on species. This also suggests that comparison of size is possible within or between species using multiple sieves. This system will particularly allow determining relative developmental status of large number of immature population with relatively less efforts without directly measuring size, volume, or weight of each individual.

The mean estimated size of immature individuals of three mosquito species were significantly different among age groups (ANOVA with Tukey HSD; $F_{7,112} = 733.5$, $P < 0.0001$) (Fig. 7.2). The results suggest that using different sieves of different opening size, different age group of immature individuals can be collected. Eggs and 1st instars were smallest, and individuals at age 96, 120, 144 hours were largest while individuals at age 24, 48 and 72 hours had different sizes one another. Based on the size estimated for each age group, sieve size can be estimated that will collect majority of each age group. For example, the size of eggs was estimated 0.210 ± 0.008mm (mean size ± SD; n = 15), thus sieve no. 70 which has nearest opening size (0.210mm) is expected to collect most of eggs. Similarly, sieve no. 70, 45, 35 and 25 can be used to collect individuals of age at 0, 24, 48 and 72 hours (Table 7.2). Sieve no. 20 can be used to collect individuals at age between 96 and 144 hours, but additional sieves around size no. 20 may reveal age specific sieve size to distinguish individuals between age 96 and 144 hours.

The sieve filtering system can be further utilized for mass rearing and sterile insect technique (SIT) based strategies for mosquitoes. Similar size of larvae can be separately grouped using different size of sieves; therefore, synchronizing the development of larvae. By utilizing sieves of different sizes, each group of larvae will have a similar size thus avoiding possible resource competition that would affect the size of the resulting pupae and adults. The sorting of females and males for mass rearing is typically performed at the pupal stage, because female pupae are usually larger than male pupae. Thus, synchronized optimal rearing of mosquitoes could aid to ensure accuracy of separation of male and female pupae for mass rearing.

This study describes the use of sieves to collect immature mosquitoes as a model filtering system of wastewater accumulated as a part of mosquito rearing process. Results suggest that the discussed sieve system can be utilized for safe and efficient disposal of eggs and immature mosquitoes, and further utilized for the mass rearing of mosquitoes. The sieves are commercially available and the filtering system can be easily constructed by stacking multiple sieves in the order of opening size. The use of multiple sieves in the filtering system will accumulate immature individuals of different sizes in particular sieves and will increase filtering efficiency by reducing potential clogging if only one small opening sieve was used. Here the system was only used for immature mosquito life stages, but could also be applied to any laboratory insects that involve aquatic stage and require a methodology to remove viable biological materials from wastewater.

**Table 7.1.** Sieve number used in the experiments and opening size

| Sieve no.* | Opening size (mm) |
|---|---|
| 10 | 2.000 |
| 20 | 0.841 |
| 40 | 0.420 |
| 60 | 0.250 |
| 80 | 0.177 |
| 100 | 0.149 |
| 120 | 0.125 |

*USA standard sieve no.

**Table 7.2.** Estimate mean size of immature age group (i.e., mean filtering opening size) and suggested sieve size to filter out the different developmental stages. For each age group, three mosquito species were combined by repeating experiments five times per strain

| Developmental stage or age (hours) | Filtering opening size (mean ± SD) | Suggested sieve no.* |
|---|---|---|
| Eggs (0 hours) | 0.210±0.009 | 70 |
| Larvae (2 hours) | 0.212±0.014 | 70 |
| Larvae (24 hours) | 0.328±0.034 | 45 |
| Larvae (48 hours) | 0.530±0.071 | 35 |
| Larvae (72 hours) | 0.709±0.073 | 25 |
| Larvae (96 hours) | 0.819±0.023 | 20 |
| Larvae (120 hours) | 0.832±0.015 | 20 |
| Larvae (144 hours) | 0.839±0.007 | 20 |

*USA standard sieve no.

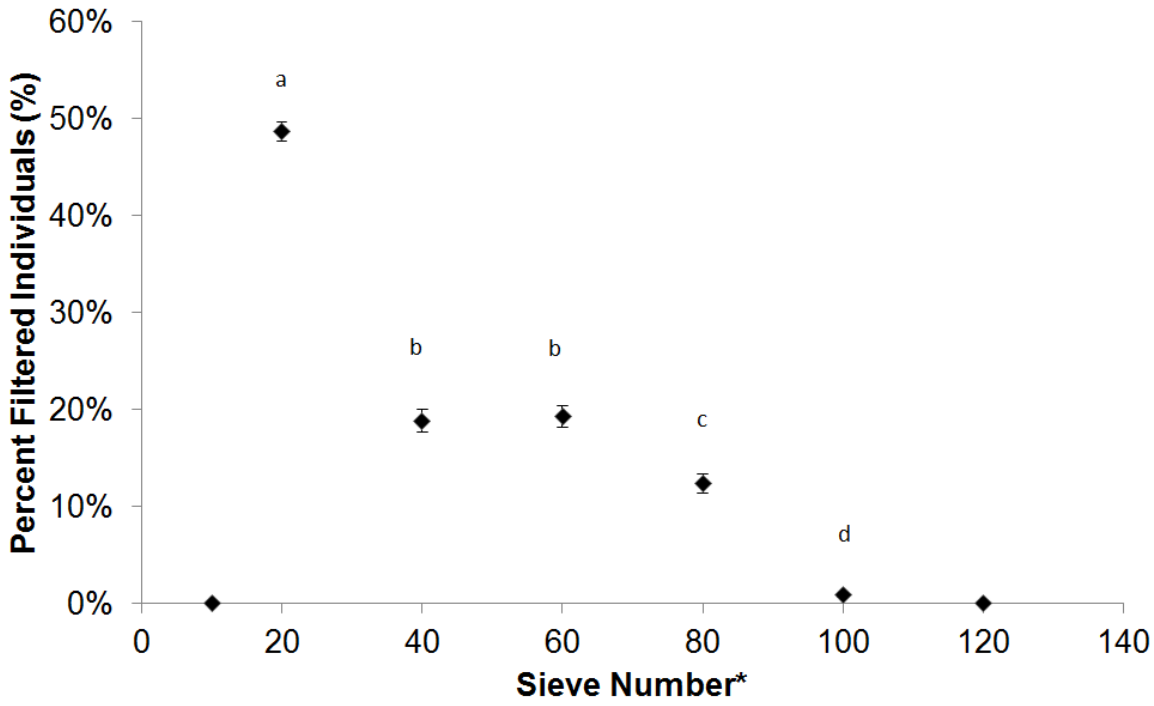


**Figure 7.1.** Combined mean proportion of APM (*A. polynsiensis*), MID (*A. albopictus*) and WACO (*A. aegypti*) filtered by sieves with different opening size. Different letters indicate significant difference at $P = 0.05$ among sieves. *Different sieve number indicates different opening size (see table 7.1). Error bar = SE (n = 15).

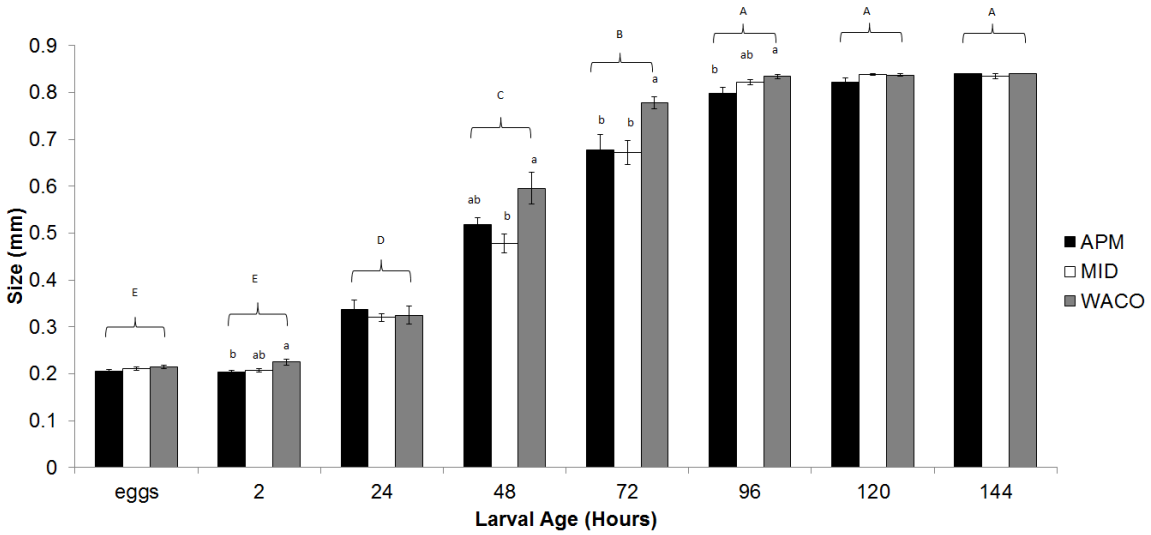


**Figure 7.2.** Estimated average size of immature mosquitoes of APM (*A. polynsiensis*), MID (*A. albopictus*) and WACO (*A. aegypti*). The mean filtering opening size of each immature group was estimated by calculating the average opening size of sieves where immature individuals were collected (i.e., sieve opening size = size of collected immature individuals). The mean filtering opening size were assumed as the size of each age group. Different letters indicate significant difference at $P = 0.05$. Error bar = SE (n = 5)

# Chapter Eight

## Artificial blood feeding system to facilitate mass rearing of mosquitoes

### Introduction

Blood meals for mosquitoes can be acquired from animal use or human volunteers. However, these methods can be limited due to ethical and regulatory concerns in addition to cost and inconvenience of breeding animals. Alternatively, blood can be artificially provided by using a membrane through which mosquitoes access the blood. In addition to the commercially available apparatus for artificial blood feeding, several feeding tools have been developed in order to reduce cost and labor in preparation and application of the tools (Tseng 2003, Mishra et al. 2005, Rampersad and Ammons 2007). However, an efficient method has not been proposed for feeding multiple cages simultaneously in order to facilitate mass rearing of mosquitoes.

Here an efficient method for artificially blood feeding multiple cages of mosquitoes is described. This method is inexpensive, disposable, and easy to use. Particularly, it allows controlling temperature of various amounts of blood and feeding mosquitoes in multiple cages with easier expandability. Furthermore as a part of this study, feeding efficiency of multiple species with the described technique was compared to a commercially available system.

### Materials and Methods

**Insect strains.** To determine applicability to multiple species, the system was tested with three wild-type aedine species. Mosquitoes used in experiments were APM strain of

*Aedes polynsiensis* (Dean and Dobson 2004), the MID strain of *A. albopictus* (Lexington, KY), and the WACO strain of *A. aegypti* (Mains et al. 2008). Mosquitoes were maintained as previously described (Dobson and Rattanadechakul 2001).

**Creation of blood bag.** A Ziploc bag (S.C. Johnson & Son Inc., Racine, WI, Canada) (18 x 22 cm) was used as a blood reservoir, and collagen (Discovery Workshops, Accrington, UK) as a feeding membrane. On one side of the Ziploc bag, a rectangular window was cut off (13 × 15cm), and the bag was temporary fixed using detachable tape to facilitate attaching the membrane to the bag (Fig. 8.1). Double sided tape (Scotch 665; St Paul, MN) was placed around the perimeter of the window and appropriate size of collagen membrane was attached from the bottom side and carefully to the top, trying not to make wrinkles or bubbles (Fig. 8.2). To avoid potential leakage of blood, the attached part of the membrane was rubbed using finger nail to remove wrinkles and bubbles, particularly on the corner of the windows where the tapes are overlapped (Fig. 8.3). About 50ml of unexpired human blood (type A+, Kentucky Blood Center, Lexington, KY) was poured into the bag and the opening of bag was zipped after removing air as much as possible in order to make a thin bag (< 0.5cm) (Fig. 8.4A).

**Blood feeding trials.** Eggs of APM, MID and WACO strain were hatched and the resulting larvae were provided liver powder solution *ad libitum.* Adult mosquitoes were eclosed and allowed to mate for 14 days. Female mosquitoes were transferred to designated cages (33.9 ± 1.4 per cage; mean ± SE; n = 18) with an access to 10% sucrose solution. A Ziploc blood bag was placed on the screen of each cage and a LCD Select Heat Heating Pad (Cara # 72; Cara Inc., Warwick, RI) was used to warm the blood bag (Fig. 8.4B) with a fixed temperature of 36.7°C determined by the thermostat attached to

the pad. A Hemotek membrane-feeding system (Discovery Workshops, Accrington, UK) was used to compare the feeding frequency of a commercially available system to the bag feeding system. A single Hemotek feeder was provided per cage following the provided protocol. The blood was accessible to mosquitoes for 60 minutes, and blood fed individuals were counted to estimate feeding frequency. Mosquitoes were considered blood fed if blood was detected inside the abdomen. Experiments were repeated three times per species.

**Statistical analysis.** The proportion of blood fed females was arcsine square root transformed to meet normality. Generalized linear model was used to test effects of mosquito species and feeding tools on feeding frequency (binomial distribution with Logit link; JMP 8.0.1; SAS Institute; Cary, NC). Chi-square test (two-tailed Fisher's Exact) was used to compare feeding frequency between Hemotek feeders and Ziploc bag feeders within the same mosquito species.

**Results and Discussion**

Parafilm has been frequently used for membrane feeders because of its low cost and wide availability as an effective feeding membrane. However, it required proper modification of the membrane (e.g., stretching) to construct blood bags (Mishra et al. 2005, Rampersad and Ammons 2007) or work as feeding membrane attaching to blood reservoirs such as petridish (Tseng 2003), which may require additional caution to avoid damaging the membrane and causing leakage of blood. Ziploc bags with collagen membrane can be more secure because collagen membrane requires no such treatment while providing more durability. The double-sided tape could securely attach collagen

membrane to the bag creating the blood bags without any leakage. Although the Ziploc bag feeder is inexpensive and disposable, it can be also reusable as long as the blood bag is preserved at 4°C until the blood is expired.

The collagen membrane also showed increased feeding efficiency. Particularly the bag system attracted more mosquitoes to the blood sources compared to parafilm-based methods of feeding trials previously conducted (data not shown here). The collagen membrane was slightly permeable to blood plasma, which presumably served as an attractant for mosquitoes on the surface of membrane.

The temperature of the blood bag was effectively maintained using the thermostat controlling the heating element in the heating pad. The previously suggested feeding tools usually required blood warming devices to circulate water in tubes connected or failed to continuously provide heating source to maintain the temperature of blood. The Ziploc bag feeders using heating pads can be easily expanded to multiple feeders by simply creating more bags and placing heating pads as the Ziploc bag feeders are separate units.

Mosquitoes could become fully engorged when feeding on the blood bag system (Fig. 8.4C). The feeding frequency of Ziploc bag feeders ranged from 20% to 80% depending on mosquito strains (Fig. 8.5). A generalized linear model test showed significant effects of mosquito species, feeding methods, and interaction effects of mosquito species × feeding methods (Table 8.1). Overall, Ziploc bag feeders showed higher feeding frequency than Hemotek feeders. Only one Hemotek feeder (surface area exposed for feeding = 9.6 $cm^2$) was used per cage due to the limitation in supplying power to multiple feeders and the area of feeding membrane was relatively small. The

wider area of feeding membrane in Ziploc bag feeders (surface area exposed for feeding = 195 $cm^2$) could have attributed to the observed increase of feeding frequency. Different sizes of Ziploc bags are available commercially, thus higher feeding frequency is expected by using larger Ziploc bags with the increased size of membrane. The Ziploc bag feeders were particularly efficient in blood feeding *A. aegypti* (WACO) resulting in 75.3 ± 2.5% (mean ± SE; n = 3) of feeding frequency, which was higher than Hemotek feeders (Chi-square test; $P < 0.0001$). *A. albopictus* (MID) showed 39.6 ± 7.9% of blood feeding frequency, significantly higher rates than Hemotek feeders ($P < 0.0001$). *A. polynesiensis* (APM) had the lowest feeding frequency (21.9 ± 3.8%) and the feeding frequency was not different from Hemotek feeders ($P = 1$). These results suggest feeding frequency can be species-specific along with feeding methods, thus additional handling (e.g., adding $CO_2$ gas to attract more mosquitoes) may be required to increase feeding frequency for a certain species such as *A. polynesiensis*.

The Ziploc blood feeding system can be constructed with all inexpensive, commonly available materials. Furthermore, because of the merits addressed above, the feeders are easily expanded to larger size and more units that will enable the transfer of the technology to feed a larger number of cages quickly and efficiently. Therefore, the system is useful in mass rearing of mosquitoes that requires blood feeding of a large number of mosquitoes.

**Table 8.1.** Generalized linear model to examine for an effect of mosquito species (APM, MID and WACO) and/or feeding methods (Hemotek and Ziploc bag feeders) on blood feeding frequency

| Factors tested | Degree of freedom | $\chi^2$ | *P* |
|---|---|---|---|
| Mosquito species | 2 | 66.97 | < 0.0001 |
| Feeding methods | 1 | 29.87 | < 0.0001 |
| Mosquito species × feeding methods | 2 | 11.95 | 0.0025 |

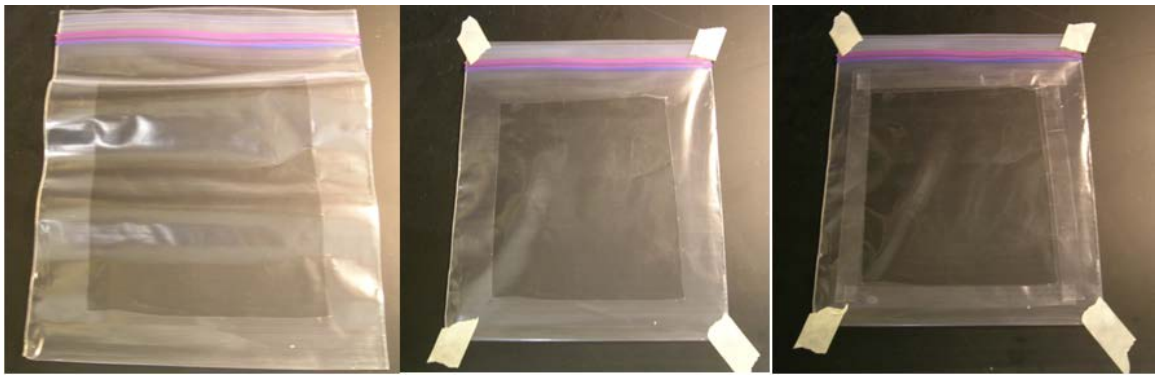

**Figure 8.1.** Double sided tape attached on the periphery parts of the window on a Ziploc bag.

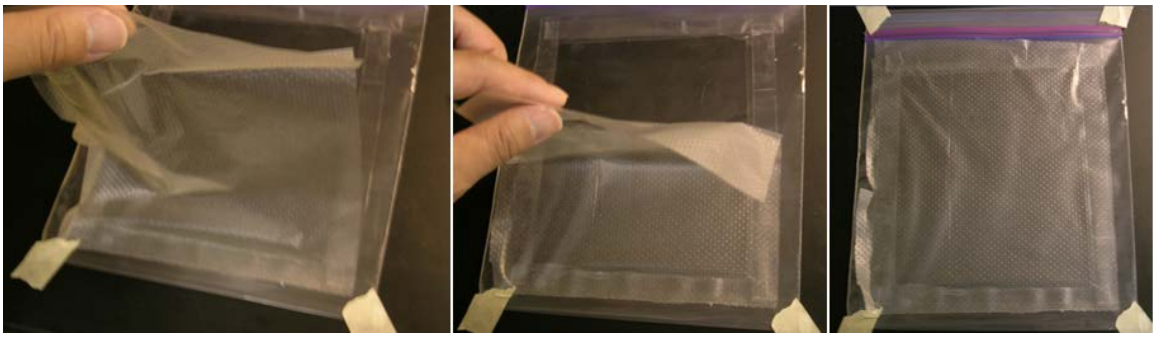

**Figure 8.2.** Collagen membrane carefully attached on double sided tapes from bottom to top on a Ziploc bag.

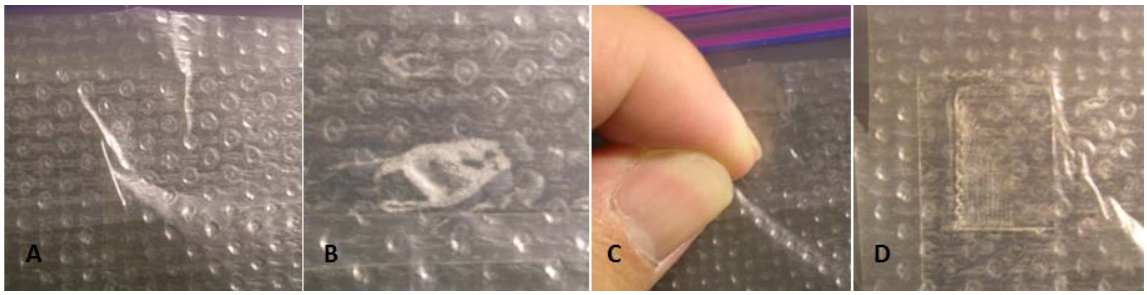


**Figure 8.3.** Wrinkles (A) and bubbles (B) removed by rubbing finger nail (C) on the attached part between tape and membrane. Overlapped part of the tapes (D) was particularly checked to avoid leakage of blood.


**Figure 8.4.** A Ziploc blood bag filled with 50ml of human blood (A) and a heating pad used to warm the blood bag (B) to feed mosquitoes that were attracted to the feeder (C).

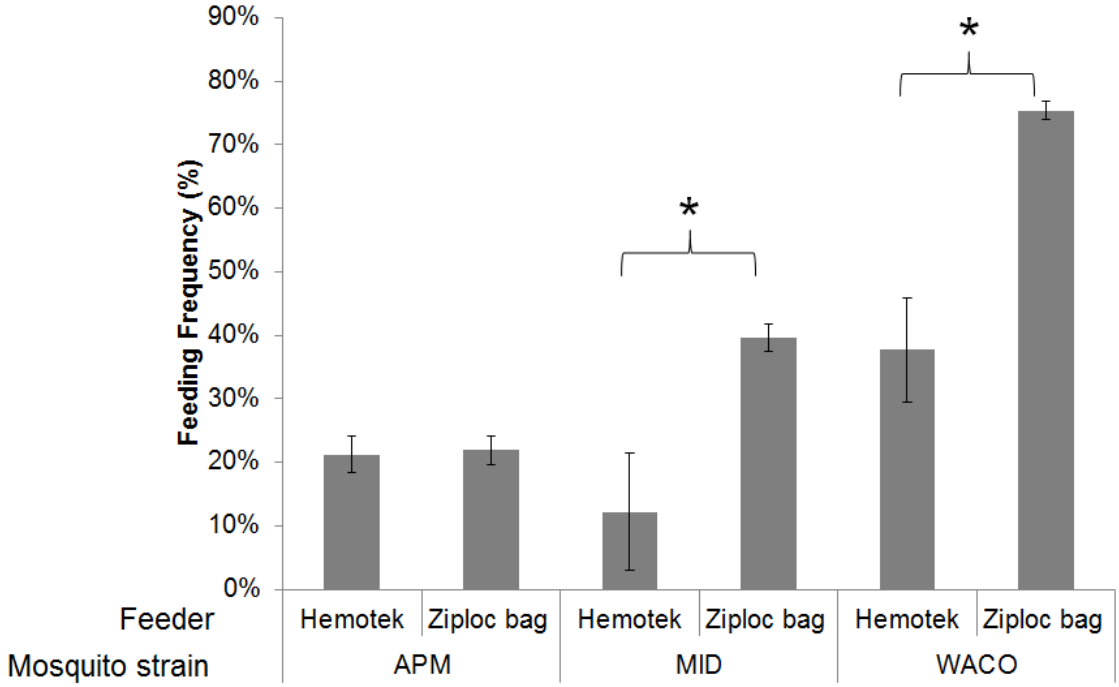


**Figure 8.5**. Blood feeding frequency of APM (*A. polynsiensis*), MID (*A. albopictus*) and WACO (*A. aegypti*) using Hemotek and Ziploc bag feeders. Asterisks represent significant difference at $P = 0.05$. Error bar = SE (n = 3).

## Chapter Nine

## Conclusion

Here, I characterize *Wolbachia* interaction with its host mosquito and examine the potential use of *Wolbachia* based control strategies for medically important mosquito vectors *Aedes aegypti* and *Aedes albopictus*. *w*MelPop is a variant strain of *Wolbachia* capable of reducing the life span of host. The life-shortening effects have made wMelPop an attractive option to use in vector control strategies. One example is the population replacement strategy that modifies the age structure of vector population to reduce disease transmission. As a part of the project, the *w*MelPop strain of *Wolbachia* was introduced into aposymbiotic *A. albopictus* and the phenotype was characterized in Chapter Two. In addition to reduced longevity and fecundity, abnormally high *Wolbachia* density was associated with embryonic mortality that masks the typical pattern of cytoplasmic incompatibility. The infection was determined to have a pathogenic association with its host, thus utilization of the *Wolbachia* infection in *A. albopictus* was not suggested for the population replacement.

*A. albopictus* harbors two *Wolbachia* strains in natural populations. In order to induce unidirectional CI for a potential population replacement, a triple infection is required. In Chapter Three, *w*MelPop infection was introduced into wild type *A. albopictus* that is naturally double infected *Wolbachia*. An unusual interaction between the *Wolbachia* infection and blood type was observed to affect host fitness, in addition to reducing the longevity of adult hosts. Unlike the single *w*MelPop infection, the triple infected individuals produced viable embryos at normal levels when the acquired blood

meal was from a human blood source. Furthermore, *Wolbachia* virulence that reduced embryo viability appeared to attenuate in the later generations of the initial *w*MelPop introduction in *A. albopictus*. The results suggested potential use in control strategies for vectors transmitting zoonotic pathogens. Further studies should focus on understanding 1) nutritional interactions between *Wolbachia*, host and blood types, and 2) *Wolbachia* interaction between zoonotic pathogens within mosquitoes. In addition, mathematical models should be constructed to describe *Wolbachia* dynamics in the population replacement use with the fitness parameters described in the aforementioned experiments.

*w*MelPop-CLA was successfully introduced into *A. aegypti* and the phenotype was described. This strain of mosquito demonstrated stable maternal inheritance, perfect CI (i.e., no hatching brood form incompatible crosses), and strong life-shortening effects. However, as a part of these characterization studies, the impact of infection cost in immature stages has not been described. Particularly, larval competitiveness can greatly affect the population dynamics of both mosquitoes and *Wolbachia*. In Chapter Four, the effects of *w*MelPop-CLA were measured on the infected host immatures that are directly competing with uninfected individuals. I observed reduced survival and delayed developmental time of *w*MelPop-CLA infected mosquitoes. In addition, the relative performance reflected by population growth rate was greatly reduced in infected immature individuals as the competition effect increased. Furthermore, the infection was associated with reduced embryonic viability over time and the virulence was more pronounced at higher temperature. Although infected males presented equal competitiveness against uninfected males in mating with uninfected females, the observed phenotype of *w*MelPop-CLA is best characterized as a pathogenic infection

causing infection cost throughout all life stages of *A. aegypti*. The results suggest a population replacement strategy will be hampered by unfavorable environments such as larval habitats with limited resources or dry season conditions that can increase embryonic mortality by suspending egg hatch. Thus, close examination of biotic and abiotic factors that may affect immature and adult development will be required for target area in the applied use of *w*MelPop-CLA infected *A. aegypti* as part of a *Wolbachia* based control strategy.

When the infected *A. aegypti* are released into the field as a part of population replacement strategy, infected larvae must compete for resources with uninfected larvae in various developmental stages in addition to immature *A. albopictus* that are commonly detected in same habitats. Chapter Five focused on the effects of the *Wolbachia* infection on larval interaction. In this chapter, I gained an understanding of the relative competitiveness of infected 1$^{st}$ instar (L1) competing with fourth instar (L4) of *A. aegypti* or *A. albopictus*. The infection was associated with reduced survival of infected L1 particularly with the presence of uninfected L4 of *A. aegypti* or *A. albopictus*. The results provided evidence of potential intra- and inter-specific predation mediated by *Wolbachia* infection. I also demonstrated that the infection was associated with modified larval behavior, which was hypothesized as a possible factor responsible for an increased susceptibility of infected L1. Thus, the *Wolbachia* infection is not beneficial to infected larvae surviving in an age structured larval population, which will be another factor that should be considered in designing a population replacement strategy. Further works can include measuring relative survival or behavior of infected L1 in more field-like condition with true predators such as *Toxorhynchites*.

According to phenotypes observed in the previous chapters, life-shortening *Wolbachia* appeared to impose fitness cost on hosts in various life stages of *A. aegypti*. Previous reports examining *w*AlbB infection in *A. aegypti* have shown an interference with pathogens in *A. aegypti*, as well as no particular cost on its host. In Chapter Six, the use of *w*AlbB infection was examined for potential vector control strategies that may be analogous to control strategies focused on *w*MelPop-CLA infections. Here, I show that with no reduction in fecundity or egg hatch, and *w*AlbB infection was bidirectionally incompatible with *w*MelPop-CLA. Results suggest possible uses of the *w*AlbB infection 1) to suppress *w*MelPop-CLA infection and 2) to utilize *w*AlbB as an additional or alternative agent for the population replacement strategy. Furthermore, I show equal mating competitiveness between the two transfected lines and no reduced CI level in old males of *w*AlbB infected *A. aegypti*. Future studies should examine for an effect of *w*AlbB infection on immature competitiveness to gain a greater understanding of *w*AlbB infection dynamics.

Studies on living modified organisms (LMO) such as *Wolbachia* infected or genetically modified insects require the prevention of the accidental release of LMOs. In Chapter Seven, a filtering system that screens immature mosquitoes is described. The system is based on filtering water used to rear immature mosquitoes using commercially available sieves. Larvae and eggs can be separated from water, which increased efficiency of disposing living mosquitoes in aquatic stage, facilitating mass rearing of mosquitoes as well as preventing release of immature individuals outside of laboratory. Further applications included using the system for an estimation of the relative size of an immature population.

Mass rearing of mosquitoes is necessary in vector control strategy such as incompatible insect technique, which requires mass release of incompatible males to suppress natural vector populations. In Chapter Eight, an artificial blood feeding system was suggested utilizing commercially available materials such as Ziploc bag, collagen membrane and heating pads. The described artificial blood feeding system is disposable and versatile for blood feeding a large number of mosquitoes. Blood feeding was successful for multiple mosquito species. Furthermore, the described system was shown to increase the numbers of mosquitoes that acquired a blood meal compared to a commercially available blood feeding system. Results suggest that the described system could be applicable for mass rearing of laboratory mosquitoes.

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