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Putting theory into practice: Predicting the invasion and stability of Wolbachia using simulation models and empirical studies

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PUTTING THEORY INTO PRACTICE: PREDICTING THE INVASION AND STABILITY OF *WOLBACHIA* USING SIMULATION MODELS AND EMPIRICAL STUDIES

DISSERTATION

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

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ABSTRACT OF DISSERTATION

PUTTING THEORY INTO PRACTICE: PREDICTING THE INVASION AND STABILITY OF *WOLBACHIA* USING SIMULATION MODELS AND EMPIRICAL STUDIES

A new strategy to fight mosquito-borne disease is based on infections of the maternally-transmitted, intracellular bacterium *Wolbachia pipientis*. Estimates predict that *Wolbachia* infects nearly half of all insect species, as well as other arthropods and some nematodes. *Wolbachia* manipulates the reproduction of its host to promote infection, most commonly causing a form of conditional sterility known as cytoplasmic incompatibility. Generally, *Wolbachia* infections are benign and do not inflict significant costs upon its host. However, studies demonstrate that some infections are associated with substantial costs to its host. These same infections can also induce pathogen interference and decrease vector competency of important disease vectors. Theory predicts that organisms that incur costs relative to conspecifics are less competitive and their competitive exclusion is expected. In the case of *Wolbachia*, the bacterium can influence reproduction such that phenotypes with lower fitness may still reach fixation in natural populations.

In this dissertation, I describe theoretical and empirical experiments that aim to understand the invasion and stability of *Wolbachia* infections that impose costs on their host. Particular attention is paid to immature insect lifestages, which have been previously marginalized. These results are discussed in relation to ongoing vector control strategies that would use *Wolbachia* to manipulate vector populations. Specifically, I discuss the cost of novel *Wolbachia* infections in *Aedes polynesiensis,* which decreases larval survival and overall fitness relative to wild-type mosquitoes. Then, a theoretical framework was developed to determine the significance of reductions in larval viability in relation to the population replacement disease control strategy. Further theoretical studies determined that *Wolbachia* infections, once established, resist re-invasion by uninfected individuals despite relatively high costs associated with infection so long as the infection produces reproductive manipulations. Additional studies determined that larvae hatched from old eggs experience reduced survival in mosquito strains with novel *Wolbachia* infections when compared to the wild-type. To validate the theoretical studies, model predictions were tested empirically to determine the importance of the larval viability. Finally, a COPAS PLUS machine was evaluated and its role in understanding

early larval development in mosquitoes is discussed. The importance of integrated research in disease control is highlighted.

KEYWORDS: Simulation model, *Wolbachia,* population replacement, population dynamics, *Aedes* mosquitoes

Philip R. Crain

Student's Signature

March 27, 2013

Date

PUTTING THEORY INTO PRACTICE: PREDICTING THE INVASION AND STABILITY OF *WOLBACHIA* USING SIMULATION MODELS AND EMPIRICAL STUDIES

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Dedicated to my wife Emily, son Beckham, father Danny and in loving memory of my mother Brenda.

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Chapter One

Introduction

Background

Arthropod-borne diseases contribute significantly to human mortality and morbidity throughout the world. Malaria, the most infamous arthropod-borne disease, killed an estimated 655,000 people in 2010 according to World Health Organization (WHO) estimates (WHO 2010). Due to recent outbreaks of other diseases, such as Dengue virus, West Nile virus, and Chikungunya, the efficacy and efficiency of arthropod-borne disease control strategies are being reevaluated (Hubalek and Halouzka 1999, Lanciotti et al. 1999, Bellini et al. 2012, Dick et al. 2012, Medlock et al. 2012). Specifically, the exponential increase in cases of Dengue virus, coupled with a worldwide distribution of its vector *Aedes aegypti*, has generated great concern (Gubler 1998, Guzman and Kouri 2002, Mackenzie et al. 2004).

Dengue virus is a member of the family Flaviviridae and is known as "breakbone fever." It causes severe flu-like symptoms including high fever, headache, and muscle and joint pain (Mullen and Durden 2002). The WHO estimates that 500,000 people require hospitalization due to infection with Dengue each year with approximately 2.5% of those cases leading to death (WHO 2012). There are four serotypes, which were geographically isolated in the recent past. However, increased human mobility and globalization has resulted in the movement of dengue virus serotypes, which now co-circulate in some regions (Rodriguez-Roche et al. 2005, Guzman et al. 2010). This is

significant because infection with two or more different serotypes increases the likelihood of Dengue Hemorrhagic Fever, a serious complication of infection with Dengue virus. Currently there is no vaccine to control Dengue virus, so effective disease control relies on controlling its vector.

Aedes aegypti is a container-breeding mosquito that utilizes both man-made and natural containers. It has a global distribution and is common in most urban and semi-urban areas throughout the tropics and sub-tropics (Focks et al. 1993a). Mosquitoes are holometabolous insects, which have four distinct life stages: egg, larva, pupa and adult (Gullan and Cranston 2005). Mosquito eggs can vary in size, shape, and strength and require deoxygenized water to begin hatching (Gjullin et al. 1941, Judson 1960). The larval stage occurs in aquatic habitats and is the major life stage affected by density dependent competition for limited resources (Dye 1984). The pupal stage is a non-feeding life stage that precedes adult emergence (Focks and Chadee 1997). Adult mosquitoes require sucrose meals after eclosion, and some female mosquitoes require bloodmeals to complete egg development (Stone et al. 2009, Stone et al. 2011). Females that take bloodmeals vector a variety of human pathogens and are the focus of disease control programs.

There are three general strategies used to control mosquito vectors. Environmental control involves limiting oviposition sites for adult females and reducing larval habitats (Heintze et al. 2007, Ballenger-Browning and Elder 2009). However, *Ae. aegypti* live in large, complex environments where environmental control may not be feasible (*i.e.,* there may be too many sites to remove). A second approach is using chemical insecticides. Mosquito control districts will employ several chemical treatments

simultaneously. Insecticide sprays, residual spraying, bed nets, and other methods are used to limit adult populations (Geetha et al. 2012, Temu et al. 2012), while larval habitats are dosed with synthetic hormones that kill mosquitoes before they emerge as adults (Shapiro et al. 1986, Fillinger et al. 2003, Devine et al. 2009). However, large-scale application of insecticides has led to occurrences of insecticide resistance and may also impact non-target species (McGaughey and Whalon 1992, Hemingway and Ranson 2000). Furthermore, spraying requires repeated application of insecticide to affect all areas, which is costly to mosquito control districts (Zaim and Guillet 2002). The final method is biological control, which is the use of one organism to reduce populations of a target species (Legner 1995). *Gambusia* sp., common name mosquitofish, can significantly decrease larval mosquito populations (Bence 1988). However, the efficacy of mosquito biological control requires that a large proportion of habitats be affected. Due to the difficulties outlined here, an additional control option is needed to help control dengue and other mosquito borne diseases.

Wolbachia pipientis is an obligate, intracellular bacterium that is found in a large proportion of insect species (Zug and Hammerstein 2012). *Wolbachia* has been identified as a possible biological control agent due to three unique attributes of its biology (Zabalou et al. 2004, Walker et al. 2011). First, *Wolbachia* is maternally transmitted with high fidelity, such that offspring of a mother infected with *Wolbachia* will receive her infection and the effects associated with it (Turelli and Hoffmann 1995, Xi et al. 2005a, McMeniman and O'Neill 2010). Second, *Wolbachia* manipulates the reproduction of its arthropod hosts to promote infection thereby acting as a gene drive mechanism (Werren

et al. 2008, Marshall 2009). Finally, *Wolbachia* infections are associated with changes in insect physiology that ultimately block human pathogens.

There are four reproductive manipulations associated with *Wolbachia* and each functions to increase infection frequency in a population. In some isopods, *Wolbachia* will feminize genetic males (Chevalier et al. 2012). In some Hymenopteran, thrips, and mite populations, *Wolbachia* induces parthenogenesis (Huigens et al. 2000, Werren et al. 2008, Stouthamer et al. 2010, Reumer et al. 2012). In lepidopterans, *Wolbachia* produces a male killing phenotype (Hornett et al. 2009, Hornett et al. 2010). However, the most common reproductive manipulation caused by *Wolbachia* is called cytoplasmic incompatibility, known as CI. CI is the reduction in egg hatch of crosses between incompatible *Wolbachia* types and can be divided into two categories, unidirectional and bidirectional CI (Dobson 2003, Werren et al. 2008). In unidirectional CI, matings between uninfected females and *Wolbachia*-infected males produce inviable eggs, effectively sterilizing those females (Figure 1.1a). In some cases, two different *Wolbachia* infection types produce unidirectional CI when crossed (Zabalou et al. 2004, Zabalou et al. 2008). For example, a superinfection, which occurs when a host is infected with two or more different *Wolbachia* infections, can be unidirectionally compatible with a singly-infected insect (Fu et al. 2010, Figure 1.1b). Alternatively, two *Wolbachia* infections may be bidirectionally incompatible where any hybrid between the two infections results in inviable embryos (Figure 1.1c).

The physiological mechanism of CI is still unknown. Multiple competing hypotheses have been generated to explain crossing patterns. The lock and key hypothesis suggests that *Wolbachia* "lock" the sperm of infected males and only females

of the same infection type have a "key" to unlock the sperm and create viable offspring (Charlat et al. 2001, Poinsot et al. 2003). The mis-timing hypothesis suggests that there is a delay during the fusion of chromosomes that produces the inviable embryo, and the delay is specific to each *Wolbachia* infection (Tram and Sullivan 2002, Ferree and Sullivan 2006). The goalkeeper hypothesis supposes a coevolutionary mechanism whereby a "modification" occurs in *Wolbachia*-infected sperm and then a "rescue" factor is selected for in females (Bossan et al. 2011). As this process continues over time, more modifications can evolve and likewise more rescue mechanisms.

Regardless the physiological mechanism of CI, the population level effects are well-known. Uninfected populations are subject to invasion by *Wolbachia*-infected conspecifics via CI (Hoffmann et al. 1990, Turelli and Hoffmann 1991). This phenomenon, known as population replacement, has been reported in nature and in laboratory studies (Turelli and Hoffmann 1991, Xi et al. 2005a, Hoffmann et al. 2011, Walker et al. 2011). Population replacement is the invasion and establishment of *Wolbachia*-infected individuals in a population that was uninfected. In an effort to better understand *Wolbachia* invasion, theoretical models were developed and determined that the invasion of *Wolbachia* is highly dependent on the maternal inheritance rate of *Wolbachia*, the level of CI, and the cost of infection on host fitness (Turelli and Hoffmann 1991, Jansen et al. 2008, Haygood and Turelli 2009, Turelli 2010, Hancock et al. 2011). Although additional studies have identified other important parameters, these three are still considered the most significant factors affecting the spread of *Wolbachia*.

Unidirectional CI is a gene drive mechanism that promotes *Wolbachia* infection within a population (Sinkins and Gould 2006, Marshall 2009). Gene drive mechanisms

are of interest to disease control researchers because these mechanisms can introduce transgenes into populations (Huang et al. 2007). Hypothetically, transgenes can be beneficial to human populations, either blocking human pathogens or eliminating vector species. However, *Wolbachia* has not been successfully transformed and therefore cannot be used to manipulate vector populations via transgenes. While *Wolbachia* has not been transformed at present, infections are associated with upregulation of insect immune genes and interference with human pathogen development (Brennan et al. 2008, Kambris et al. 2009, Moreira et al. 2009, Bian et al. 2010, Kambris et al. 2010, Hughes et al. 2011, Pan et al. 2012, Rances et al. 2012, Weiss et al. 2012). *Aedes aegypti,* the primary vector of Dengue virus, has been artificially infected with several *Wolbachia* infections and those infections are associated with decreases in Dengue virus titer (Walker et al. 2011, Pan et al. 2012). Similarly, there are significant decreases in Chikungunya virus titer when *Aedes aegypti* is artificially infected with *Wolbachia* (Moreira et al. 2009). However, *Wolbachia* also limits non-viral pathogens. In the vector species *Aedes polynesiensis* and *Aedes aegypti*, artificial *Wolbachia* infections decrease the number of infective filarial nematodes present after 10 days (Kambris et al. 2009, Andrews et al. 2012).

Pathogen interference, typically associated with novel *Wolbachia* infections that have been introduced into a new host, activates the insect immune system (Brennan et al. 2008, Kambris et al. 2009, Bian et al. 2010, Kambris et al. 2010, Hughes et al. 2011, Pan et al. 2012). Currently, two mechanisms can be used to transfer *Wolbachia* infections from one host to another. The first mechanism, called microinjection, removes the cytoplasm from a *Wolbachia* infected egg which is then injected into an uninfected egg

(Xi et al. 2005b). Although the success rate of microinjection is low, stable infections are produced (Andrews et al. 2012). A second mechanism is through interspecific introgression crosses (Brelsfoard et al. 2008). Here, a female with the desired *Wolbachia* infection is backcrossed with males from the target strain until an insect is produced with a new *Wolbachia* infection and the same target genotype. Examples of artificial infections are found in several insect species, and several different infections have been moved with some success (Xi et al. 2005a, McMeniman et al. 2009).

Wolbachia is being evaluated as a biological control agent because it can invade populations and limit disease after being introduced into disease vectors. Two different biological control strategies utilizing *Wolbachia* have been suggested. The first is an extension of the incompatible insect technique (IIT) called population suppression (Figure 1.2a) and is focused on the mass release of male insects with different *Wolbachia* types compared to the wild-type females (O'Connor et al. 2012). These males seek out females and mate with them, effectively sterilizing them (via CI-inducing *Wolbachia*). The second technique, known as population replacement (Figure 1.2b), releases female insects with a different *Wolbachia* type into the wild-type population (Hoffmann et al. 2011). This strategy can only be successful if the released *Wolbachia* infection causes unidirectional CI with the indigenous, wild-type population.. Population replacement is not a new concept (Marshall 2009), but the biology of *Wolbachia* may eliminate the need for a transgene, *i.e.* a gene originating from another organism that has been introduced using recombinant techniques. In the case of *Wolbachia*, proponents emphasize that disease control can be obtained through a population replacement event using natural *Wolbachia* types that limit pathogen development without additional genetic

modification. This eliminates a major hurdle in gene drive theory, *i.e.* over time a gene driver and its transgene will likely become unlinked, at which time the transgene will be lost from the population (Huang et al. 2007).

However, there is a need to understand additional factors influencing population replacement (Rasgon and Scott 2004). Previous studies have increased our understanding of population replacement both theoretically and empirically (Turelli and Hoffmann 1991, Xi et al. 2005a, Walker et al. 2011), and studies have identified parameters important to population replacement (Turelli 2010, Crain et al. 2011). However, with the exception of a few studies (Dobson et al. 2002b, Rasgon and Scott 2004), the population dynamics of hosts infected with *Wolbachia* have not been considered. Empirically, cage studies have been performed that have documented population replacement (Xi et al. 2005a, Walker et al. 2011), but currently these lack realistic population dynamics. However, a real world example has seen uninfected populations of mosquitoes in two Australian towns replaced by *Wolbachia* infected released mosquitoes (Hoffmann et al. 2011). Despite these empirical and theoretical considerations, there is still a lack of understanding concerning what factors dictate population replacement and how sensitive the success of invasion is on those parameters. Further, a unifying theoretical framework that facilitates empirical studies is needed.

Specific Aims/Objectives

The dissertation presented here will evaluate the use of *Wolbachia* to achieve population replacement both theoretically and empirically. Experiments were conceptualized and evaluated in a broader theoretical framework. Specifically,

preliminary experiments were designed to characterize the mosquito-*Wolbachia* system, which led to the development of a simulation model. Further empirical experiments were developed to assess hypotheses generated by model predictions.

Initial experiments were designed to characterize the effects of novel *Wolbachia* infections on mosquito hosts. These experiments were designed to determine differences in mosquito fitness and involved building life tables for several mosquito strains. Additionally, the the patterns of CI between these strains were characterized. These results were used to construct a computer simulation model that described mosquito population dynamics and *Wolbachia* infection dynamics. New parameters were identified during model development and their relative importance to population replacement was discussed. The model was later modified to address a specific disease control program that is ongoing in Australia. The model was modified to include multiple, competing infection types, and the dynamics of competition between two *Wolbachia* strains was analyzed. Finally, the model was modified again to evaluate the stability of *Wolbachia* infections once a population has become infected. Here, uninfected individuals are released into *Wolbachia* infected populations to assess reverse population replacement. Empirical assays were then designed to test theoretical predictions regarding population replacement. Specifically, different initial infection frequencies were initialized and the change in infection level over one generation was calculated, factoring in differences in net reproductive rate. Another empirical assay identified a mechanism whereby larval viability can be reduced when larvae hatch from eggs that have been dormant for long periods of time. Finally, a large scale flow cytometer was examined as a tool to understand development in first instar mosquito larvae. All results are discussed in a

broader framework and look to reconcile specific results with overall disease control tactics.

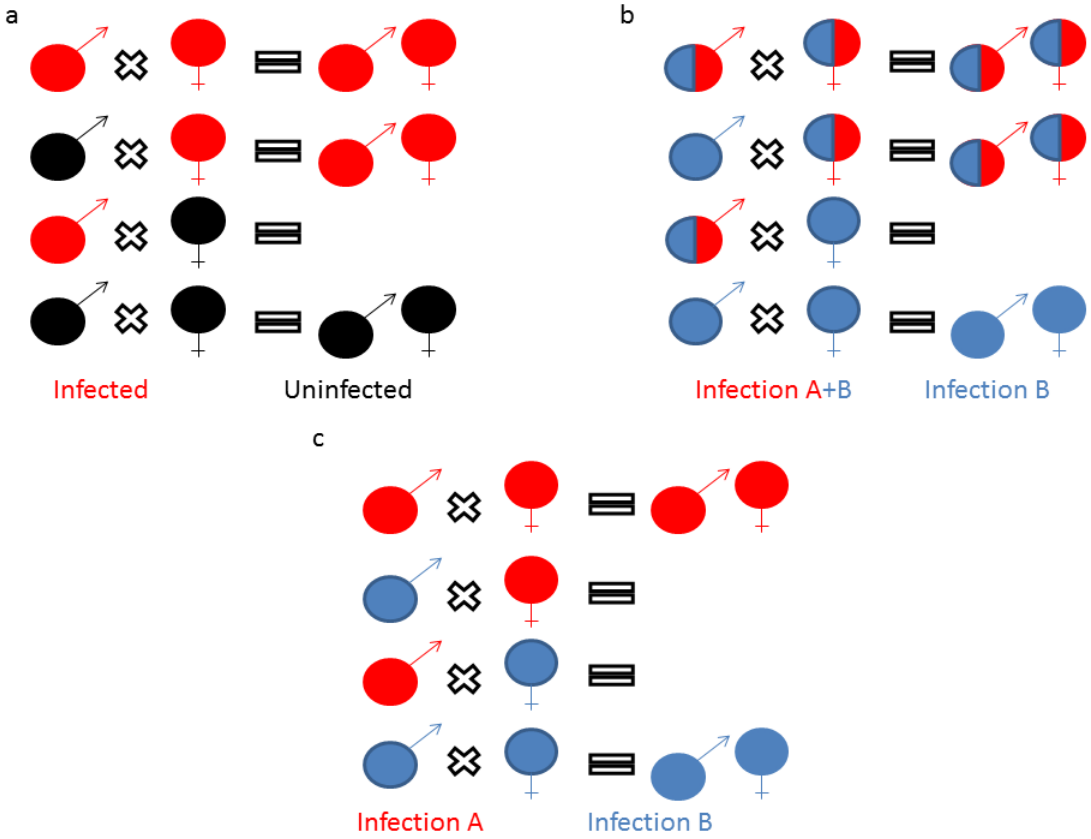


Figure 1.1. Description of the patterns of cytoplasmic incompatibility, CI, caused by infection with *Wolbachia*. a) Unidirectional CI occurs when uninfected females (black) mate with *Wolbachia* infected males (red). This cross is termed an incompatible cross because no viable embryos are produced, which gives *Wolbachia* infected females an advantage relative to uninfected females. b) Unidirectional CI can also occur when superinfected (two or more *Wolbachia* infections coexist within one host) individuals mate with singly infected individuals. c) Bidirectional CI occurs when two individuals mate and both have a different *Wolbachia* infection. Hybrid crosses between the two infection types result in incompatible crosses.

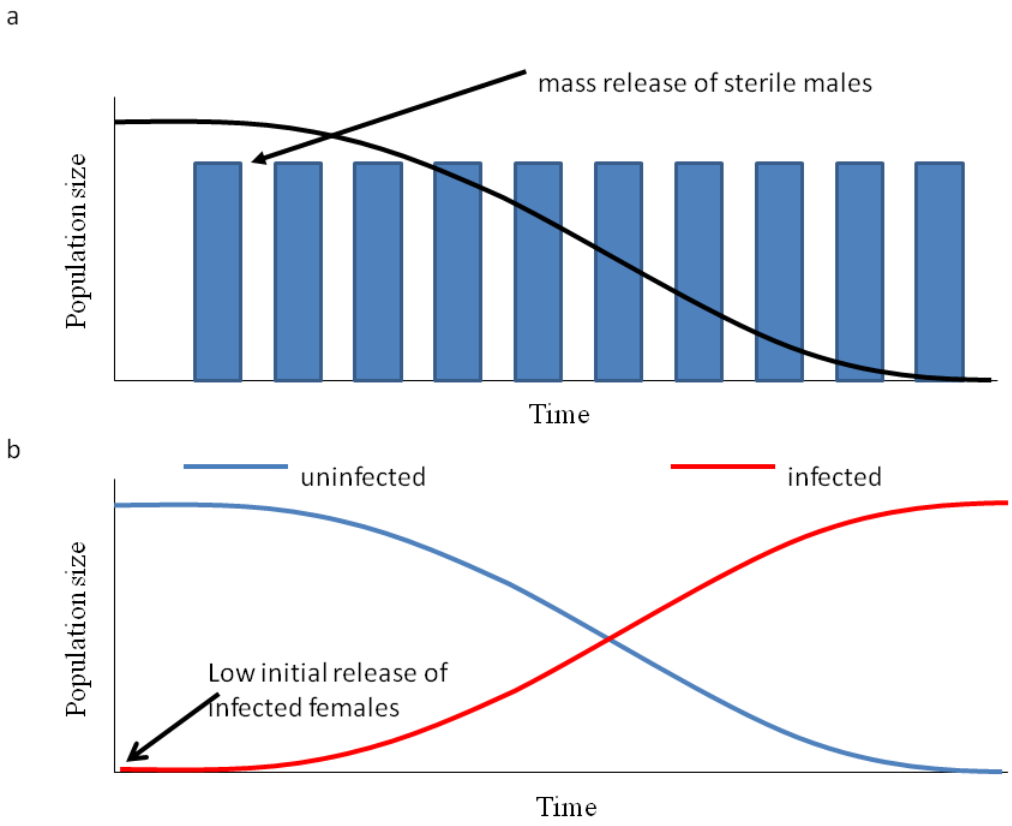


Figure 1.2. Diagram of proposed biological control strategies using *Wolbachia* infected individuals. a) Population suppression is a *Wolbachia*-based vector control strategy that is a form of incompatible insect technique (IIT). In IIT programs, there are multiple inundative releases of incompatible (functionally sterile) males. By releasing many males, the goal of this program is to sterilize wild-type females thereby lowering population densities in areas with large insect pest populations. b) Population replacement is an inoculative release program where infected female insects are released into an uninfected population, or a unidirectionally compatible population. Here, the females mate with males in the wild-type population successfully and infection spreads through vertical transmission and later through incompatible matings. The goal of

population replacement is not to decrease population densities, but rather change the infection status of an insect population.

Chapter Two

Artificial *Wolbachia* infections decrease net reproductive rate in the vector mosquito species *Aedes polynesiensis*

Introduction

Wolbachia pipientis is a maternally-inherited, intracellular bacterium that manipulates arthropod reproduction to promote infection. Infections of *Wolbachia* are common, with current estimates suggesting that 40% of all terrestrial arthropod species are infected (Zug and Hammerstein 2012). *Wolbachia* is associated with four reproductive manipulations, specifically: feminization of genetic males (Chevalier et al. 2012), parthenogenesis (Reumer et al. 2012), male-killing (Hornett et al. 2010) and cytoplasmic incompatibility (Caspari and Watson 1959). Cytoplasmic incompatibility, known as CI, is the most common form of reproductive manipulation (Werren et al. 2008). In brief, CI results in karyogamy failure in crosses between uninfected females and infected males, thus these "incompatible crosses" produce no viable offspring (Werren 1997). CI creates a reproductive advantage for *Wolbachia*-infected females who can mate with either infected or uninfected males in a population. Therefore, *Wolbachia* infections are predicted to increase in frequency to fixation when introduced into uninfected populations (Turelli and Hoffmann 1991).

Lymphatic filariasis (LF) is a global disease that currently affects 120 million people with an estimated 1.3 billion at risk of infection (World Health Organization 2012). LF is caused by nematodes, predominantly *Wuchereria bancrofti*, and is vectored by several mosquito genera (Ottesen 2000). LF has been recognized as an eradicable

disease, and currently a mass drug administration (MDA) program has been implemented with some success (Esterre et al. 2005, Gass et al. 2012). In the South Pacific, success of the MDA strategy is complicated in part by the biology of the primary vector species *Aedes polynesiensis*. MDA programs reduce the number of nematodes in the human population through antibiotics, but *Ae. polynesiensis* becomes a more efficient vector when fewer nematodes are present (Southgate 1992). This process, known as limitation, compromises the MDA strategy (Pichon 2002). Thus, alternate control methods are needed to help eliminate LF in the South Pacific.

Wolbachia is being developed as a biological control agent for pest populations. Currently, two control strategies using *Wolbachia* have been proposed. The first, termed "population replacement," uses *Wolbachia* as a gene drive mechanism, such that uninfected population are invaded by *Wolbachia*-infected individuals (Marshall 2009). However, this strategy has limited application because it requires a unidirectional *Wolbachia* infection that is compatible with the wild-type population. A second control strategy using *Wolbachia*, termed "population suppression," can be used to target both infected and uninfected populations (Chambers et al. 2011). Population suppression is an incompatible insect technique, which requires the inundative release of male mosquitoes with *Wolbachia* infections that are incompatible with target populations. Matings between the target population and the released males result in sterilization of wild-type females, which can reduce mosquito populations. Importantly, new *Wolbachia* infections can be generated by using two techniques: introgression crosses (Brelsfoard et al. 2008) or microinjection (Xi et al. 2005a). By utilizing either technique, "novel" mosquito strains can be generated for population suppression.

As part of a population suppression control strategy, the risk of population replacement, which can occur upon accidental release of female mosquitoes, should be considered (Dobson et al. 2002b, Xi et al. 2005a, Brelsfoard et al. 2009). The risk of accidental population replacement is determined by several factors, chiefly the number of females released, the total target population size, and the relative fitness of the released females. Some theoretical studies have attempted to estimate the probability of population replacement given the release of a single female (Rigaud and Rousset 1996, Jansen et al. 2008), and these studies predict that those probabilities are low, but can be increased if relative fitness is higher in the released strain. Therefore, estimating relative fitness, determined by life table analyses, can help determine if particular *Wolbachia* infections would establish upon accidental release (Sarakatsanou et al. 2011).

In the present study, I consider the effects of artificial *Wolbachia* infections on the fitness of *Aedes polynesiensis* mosquitoes. Life table analyses are used to estimate the net reproductive rate of three mosquito strains, each with a different *Wolbachia* infection. The results are then used to evaluate the efficacy of the strains as biological control candidates, but also to understand the complex symbiosis of *Wolbachia* and its host.

Methods

Mosquitoes:

Three strains of *Aedes polynesiensis* mosquitoes were used in this experiment. A wild-type strain, designated as APM, was collected from Maupiti, French Polynesia and has been maintained as a laboratory colony for greater than five years (Dean and Dobson 2004, Brelsfoard and Dobson 2012). APM mosquitoes are naturally infected with *w*PolA,

an A-clade *Wolbachia* infection (Dean and Dobson 2004, Brelsfoard et al. 2008). The second strain, designated CP, resulted from a series of introgression crosses between *Aedes riversi* and the *Ae. polynesiensis* strain APM. CP is genetically similar to the wild-type strain, but has the *w*RivB B-clade *Wolbachia* infection (Brelsfoard et al. 2008). The MTB mosquito strain was created by the microinjection of *Aedes albopictus* cytoplasm into tetracycline-treated (aposymbiotic) APM eggs (Andrews et al. 2012). MTB mosquitoes have the same host background as APM and CP mosquitoes, but have a different B-clade infection, *w*AlbB, than CP.

Experiment 1 Bioassay:

Eggs were hatched by submersion in dilute liver powder solution of 0.6g/L (MP Biomedicals LLC, Solon, OH) for two hours. 100 first instar larvae were collected from each of the three strains using a 14.6-cm Pasteur Pipette (BD Biosciences, Franklin Lakes, NJ) and transferred into 21 x 21 x 7.5-cm plastic containers (Pactive, Lake Forest, IL) containing 40mL of liver powder solution and 460 mL of distilled water. 200mg of dried liver powder was added to the rearing container. Prior to its addition, the 40mL of liver powder solution was placed in a centrifuge to separate solution from sediment. The resulting supernatant was added to the larval rearing container. After 48 hours, an additional 200mg of dried liver powder was added to each rearing container. Larvae were allowed to pupate, and all pupae were removed and transferred to 13 x 100-mm test tubes (Fisher, Pittsburgh, PA) which were filled with distilled, deionized water. Pupae remained in test tubes until adult emergence. From each strain, 10 adult males and 10 adult females were introduced into a cage and allowed to mate. In each cage, mosquitoes

were given a 10% sucrose solution. Once per week, an anesthetized mouse was provided for females to acquire a bloodmeal (IACUC #00905A2005). After the first bloodmeal, an oviposition cup, consisting of a 100-mL specimen cup filled with DI water and lined with germination paper (Anchor Paper, St. Paul, MN), was added to each cage for female oviposition. Egg papers were replaced weekly, and the removed egg papers were allowed to embryonate in insectary conditions (~27°C and 80% RH, 18:6 light:dark cycle) for seven days. After a seven-day incubation period, eggs were submerged in dilute liver powder solution for two days. The number of eggs on each paper were counted and scored as hatched or unhatched depending on the position of the operculum. Dead adult mosquitoes were collected once per week, counted and identified to sex. Longevity was recorded until the last adult died.

Experiment 2 Bioassay:

A second bioassay was designed to more precisely estimate net reproductive rate for each of the three strains. All procedures were repeated as discussed above except for several minor changes. When larvae from each strain were moved into separate rearing containers, each rearing pan contained 500mL of distilled, deionized water and 200mg of dry liver powder (the addition of centrifuged liver powder solution was omitted). All emerging adults were moved to a cage (12" x 12" x 12" lumite screen cage, BioQuip Products, CA, USA), instead of the original 10 individuals per sex. The number of mosquitoes within each cage varied (mean number adults/cage ± SD: APM, ♀ = 35.0±6.6, ♂ = 46.7±3.5; CP, ♀ = 20.7±2.9, ♂ = 17.3±9.7; MTB, ♀ = 22.0±1.7, ♂ =

13.7±2.1). Adult mosquitoes were treated as described in the experiment one bioassay section. Experiment two was replicated three times.

Crossing experiment

A series of crosses between all three strains were designed to evaluate the CI pattern between the different *Wolbachia* strains. In these experiments, mosquitoes were reared with abundant food and low competition. To assure virginity, pupae were isolated in culture tubes and held until emergence. The sex of all emerging adults was recorded and all adults were added to crossing cages. Each crossing cage was initiated with 10 male and 10 female mosquitoes. Males and females were allowed to mate in the cages, and then females were provided a mouse once for 20 minutes to obtain a bloodmeal, after which oviposition cups were added to each crossing cage. Females were given one week to oviposit, and then egg cups were removed and stored for another week in insectary conditions. After storage, egg papers were hatched in dilute liver powder solution for two days. To determine the hatch rate, egg papers were then removed and eggs were scored as hatched or unhatched.

Data and statistical analyses:

For both experimental bioassays, five response variables were collected to compare immature mosquito life history parameters. For each strain, the median time for larvae to reach pupation, the proportion of larvae surviving to pupation, the median time from pupation to adult emergence, the proportion of pupae surviving to adult emergence, and the sex ratio was recorded. These were all proportional data, thus were arcsine-square

root transformed to achieve normality. To test for an overall difference in immature life histories, all variables were analyzed by a MANOVA using JMP 9.0 (2010 SAS Institute, NC, USA). Three factors were identified in the MANOVA, specifically: STRAIN, which consisted of three levels, the wild-type (APM), introgressed (CP) or microinjected (MTB) mosquito strains; REPLICATE, which had three levels and was included to rule out significant differences over replicate trials; and EXPERIMENT, which had two levels and was included to control for differences between the designs of the bioassays. Insignificant factors were dropped from analysis, and each significant variable was analyzed individually by an ANOVA. Post-hoc Tukey HSD or Student's t-tests were used for mean separation if applicable. A Student's t-test was used when a factor had two levels, and a Tukey HSD test was used if a factor had more than two levels.

To determine if there were differences in adult life history parameters, two response variables were collected, the egg hatch rate and the number of eggs oviposited per female. These parameters were analyzed separately from the previously described immature life history parameters because an additional factor was included in analysis. Egg hatch was recorded as a proportion of eggs hatching and was arcsine-square root transformed to achieve normality. Both response variables were analyzed in a MANOVA with STRAIN, REPLICATE and EXPERIMENT as factors, but also included gonotrophic cycle as an additional factor (denoted by CYCLE). Insignificant factors were dropped from analysis and significant response variables were analyzed individually by an ANOVA. Post-hoc Tukey HSD or Student's t-tests were used for mean separation. Longevity data was analyzed separately by a Cox Proportional Hazard test, with STRAIN, EXPERIMENT, REPLICATE and SEX as factors.

Due to the discrete nature of the experiment (*i.e.*, single, non-overlapping generations were considered), life tables were constructed and the net reproductive rate (R_0) was compared between strains. Following the first gonotrophic cycle, eggs from each strain were hatched, and 100 first instar larvae were reared to adulthood to estimate F1 larval-adult survival and sex ratio. To estimate the total number of expected reproductive individuals in the next generation, the number of hatched eggs for each replicate was multiplied by the larval-adult survival rate and the proportion of female offspring. R_0 was calculated by taking the sum of all $l_x m_x$ products (see Table 2.1). To assess differences in R_0, the net reproductive rate of each strain was averaged across replicates and analyzed by an ANOVA with STRAIN, EXPERIMENT, and REPLICATE as factors.

The hatch rates among different crosses were analyzed by a Mann-Whitney U-Test. Due to CI, some crosses had no hatching eggs which resulted in a non-normal distribution. Means separation was performed by using the Steel-Dwass all pairs comparison for nonparametric data.

Results

Immature life history parameters

There was a significant difference in immature life history parameters in the data collected from both bioassays (Wilks' Lambda=0.007, F=3.44, $p < 0.001$). STRAIN (Wilks' Lamdba=0.143, F=2.64, $p < 0.05$) and EXPERIMENT (F Test=9.833, F=15.73, $p < 0.001$) were significant factors, but REPLICATE was not significant (Wilks' Lambda=0.333, F=1.17, $p = 0.374$) and was dropped from subsequent analyses.

Due to high correlation between response variables for immature life history parameters, additional statistical tests were used to eliminate repetitive variables. There was no significant difference in the duration of the pupal stage ($F_{3,14}$=2.72, p = 0.080, where duration of the pupal stage is median emergence time – median pupation time), therefore only median pupation time was analyzed by ANOVA. There was a significant difference in median pupation time ($F_{3,14}$=23.84, p < 0.0001). STRAIN (*i.e.*, wild-type, introgressed or microinjected) was not a significant factor ($F_{2,14}$=1.76, p = 0.21), but EXPERIMENT was significant ($F_{1,14}$=68.01, p < 0.0001; Figure 2.1). Larvae in experiment one reached pupation earlier than larvae in experiment two (134.13 ± 10.57 hours compared to 166.15 ± 6.06 hours respectively, mean ± SD).

The rate of pupal failure, defined as the proportion of mosquitoes that die during the pupal life stage, was indistinguishable among strains and across experiments ($F_{3,14}$=0.82, p = 0.50). Therefore, only larval to adult survival was analyzed statistically. There was a significant difference in larval to adult survival ($F_{3,14}$=7.34, p < 0.005). EXPERIMENT was not significant ($F_{1,14}$=0.66, p = 0.43), but STRAIN had a significant effect on survival ($F_{2,14}$=10.62, p < 0.005). A post-hoc Tukey HSD test determined APM had significantly higher larval-adult survival than CP and MTB which had equivalent survival (Figure 2.2).

There was no significant difference detected by ANOVA for sex ratio, which was measured as the proportion of female mosquitoes ($F_{3,14}$=1.81, p = 0.19).

Adult life history parameters

A MANOVA was designed with STRAIN, REPLICATE, EXPERIMENT and gonotrophic cycle (identified as CYCLE henceforth), as factors explaining two response variables: per female fecundity and egg hatch rate. The overall response was significant (Wilks' Lambda=0.393, F=2.77, p<0.001), but only STRAIN (Wilks' Lambda=0.746, F=3.32, p<0.05) and CYCLE (Wilks' Lambda=0.919, F=0.91, p<0.0001) were identified as significant factors. Further analyses did not include insignificant factors (*i.e.*, REPLICATE and EXPERIMENT).

The number of eggs laid per female was not significantly different ($F_{4,48}$=1.04, p = 0.08), but there was significant difference in hatch rate ($F_{4,48}$=9.49, $p < 0.0001$). Hatch rate differed by STRAIN ($F_{2,48}$=6.35, $p < 0.005$) and by CYCLE ($F_{2,48}$=12.01, $p < 0.0001$). Wild-type (APM) and microinjected (MTB) mosquitoes had comparable hatch rates (Figure 2.3), as did microinjected and introgressed (CP) mosquitoes, but wild-type mosquitoes had a significantly higher hatch rate than the introgressed line (Figure 2.3). Hatch rate was also significantly higher in the first gonotrophic cycle compared to later gonotrophic cycles.

Adult Longevity

A Cox Proportional Hazard test determined there were significant differences in longevity (χ^2_6=38.57, $p < 0.0001$). REPLICATE (χ^2_2 = 33.29, $p < 0.001$) and EXPERIMENT (χ^2_1 = 6.03, $p < 0.05$) were significantly different, however there were no differences in longevity detected by STRAIN (χ^2_2 = 1.66, p = 0.44, Figure 2.4) or SEX (χ^2_1 = 0.03, p = 0.86).

Life table comparison

To compare net reproductive rate, R_0 , an ANOVA was conducted with STRAIN, EXPERIMENT and REPLICATE as factors, and a significant difference was identified ($F_{5,12}$=3.95, $p < 0.05$). Neither EXPERIMENT ($F_{1,14}$=0.002, $p = 0.97$) nor REPLICATE ($F_{2,14}$=1.67, $p = 0.23$) significantly affected net reproductive rate. However, there was a significant difference with STRAIN ($F_{2,14}$=13.48, $p < 0.01$). A post-hoc Tukey HSD test determined wild-type mosquitoes (APM) had a significantly higher net reproductive rate than either introgressed (CP) or microinjected (MTB) mosquitoes, which were equivalent (Figure 2.5).

Crossing experiment

There was a statistical difference between the hatch rates across all crosses (χ^2_4 = 29.17, $p < 0.001$). Means separation via the Steel-Dwass all pairs method determined that two novel crosses were significantly higher than the other three (Table 2.2). MTB x MTB crosses (female x male) and CP x MTB crosses had a significantly higher hatch rate than APM x MTB, MTB x APM, and MTB x CP crosses (Table 2.2).

Discussion

Artificial *Wolbachia* infections can be associated with a decrease in various life history parameters, ultimately affecting net reproductive rate. Regardless of the method of *Wolbachia* transfer (*i.e.*, introgression or microinjection), artificially infected strains were less fit than wild-type mosquitoes. By comparing life tables among the strains, the

lower net reproductive rate is associated with reduced larval survival in artificially infected mosquitoes. Other parameters affecting fitness, for example per capita female fecundity, were not significantly different between the mosquito strains, except for hatch rate, which was significantly lower in introgressed strains relative to the wild-type. Although the physiological cause of this reduction in fitness is not understood, such negative effects have the potential to affect pest control tactics that implement *Wolbachia*.

Wolbachia infections are known to cause reproductive manipulations in their host, but recently, novel *Wolbachia* infections have been associated with physiological change in their host (Werren et al. 2008, Walker et al. 2011, Pan et al. 2012). *Wolbachia* infections that are not co-evolved with their host can be associated with increases in reactive oxygen species levels, the upregulation of genes in insect immune pathways, and can increase virus resistance in its host (Brennan et al. 2008, Pan et al. 2012). The presence of artificial *Wolbachia* primes the immune system which is hypothesized to lead to anti-pathogen properties (Moreira et al. 2009, Bian et al. 2010, Kambris et al. 2010, Rances et al. 2012, Vavre and Charlat 2012). The increase in resource allocation to immunity likely has negative effects on the host, via tradeoffs (Tate and Rudolf 2012). In a species of field cricket, increases in immune enzymes are associated with negative life history effects (Jacot et al. 2004, Jacot et al. 2005). In *Drosophila*, the *w*Ri *Wolbachia* infection was originally parasitic, but has coevolved with its host where it is now a mutualist (Weeks et al. 2007). Furthermore, the long-term serial passage of *w*MelPop (a pathogenic strain of *Wolbachia*) in mosquito cell lines resulted in an infection that was less pathogenic than the non-cell line adapted strain (McMeniman et al. 2008). Such

studies suggest that the costs associated with artificial *Wolbachia* infections may be transient over evolutionary time. Therefore, long term, multigenerational experiments that track fitness could help determine if costly infections become less pathogenic over time.

Despite the negative effects on host fitness associated with many artificial *Wolbachia* infections, *Wolbachia* remains a viable biological control candidate. Recent field trials in Australia have shown that mosquitoes with artificial *Wolbachia* infections can replace uninfected, wild-type mosquito populations (Hoffmann et al. 2011). However, the field releases in Australia involved releases of *Wolbachia*-infected individuals into *Aedes aegypti* populations, which are naturally *Wolbachia*-uninfected. *Aedes polynesiensis* is naturally infected with an A-type *Wolbachia,* and both novel strains discussed here are infected with B-type *Wolbachia* infections (Brelsfoard et al. 2008, Andrews et al. 2012). Generally, crosses between two individuals with different *Wolbachia* infections are predicted to be bidirectionally incompatible, resulting in inviable embryos (Ahantarig and Kittayapong 2011). Bidirectional CI inhibits population replacement, but facilitates population suppression (Chambers et al. 2011). Population suppression involves inundative releases of male mosquitoes with a different *Wolbachia* infection relative to the wild-type females, which "sterilizes" the females in the population thus reducing the number of adult mosquitoes. In population suppression, the release of artificially infected females is not desirable. Data presented here suggest both strains would be viable for incompatible insect technique strategies because each strain induces CI and reduces the fitness of the artificially infected strains, reducing the opportunity for accidental population replacement.

Aside from its importance as a biological control agent of pest organisms, *Wolbachia* is recognized as an important microorganism in general ecology. Theoretical studies predict that CI-inducing *Wolbachia* could facilitate speciation (Telschow et al. 2007). Furthermore, natural population replacement of an infected biotype into an uninfected population has been described (Turelli and Hoffmann 1991). However, data presented here present an ecological paradox. Specifically, while life history characteristics favor one strain, it is plausible that the CI phenotype associated with *Wolbachia* could favor another strain. Theoretical studies have examined the likelihood of population replacement when both *Wolbachia* and life history parameters are varied and predict that costly infections have low probabilities of reaching fixation in a population (Crain et al. 2011). Most newly-introduced *Wolbachia* infections are associated with physiological change in its host, which can negatively affect life history characteristics (Jacot et al. 2004, Jacot et al. 2005, Moreira et al. 2009). Thus, how can new *Wolbachia* infections spread to fixation through uninfected populations given the negative effects associated with infection? Infection with *Wolbachia* is associated with reduction in virus-induced mortality in some insect species (Hedges et al. 2008, Teixeira et al. 2008, Osborne et al. 2009). By providing virus protection, *Wolbachia* can spread into uninfected host populations (Fenton et al. 2011). Furthermore, the invasion of *Wolbachia* into smaller populations which are linked to larger populations via gene flow could facilitate the spread of infection (Reuter et al. 2008, Barton and Turelli 2011). Future experiments should examine if the *w*AlbB infection in *Aedes polynesiensis* has positive effects, such as increased viral resistance. If such properties exist, extra attention should be given to field releases to assure no female mosquitoes are released.

Bidirectional CI results from matings between individuals with different *Wolbachia* types (Telschow et al. 2005). However, some *Wolbachia* infections contain multiple rescue factors, which allow them to mate successfully with other *Wolbachia*-infected individuals, bypassing the effects of CI (Zabalou et al. 2008). Interestingly, some crosses between different *Ae. polynesiensis* strains considered here demonstrate unidirectional CI. From an ecological perspective, such infections have a relative, reproductive advantage when compared with other infections. By increasing the number of potential compatible matings, it is hypothesized that such infections would increase to fixation in a population. Future theoretical studies could compare the three infections described here to evaluate the population and infection dynamics. However, from a biological control perspective, strains that can rescue other infections could compromise IIT strategies. To eliminate pest populations, released males need to sterilize wild-type females. Releasing a unidirectionally compatible strain would not affect target populations.

Important to the rapid development of larvae is the availability of nutrients. For example, bacteria are an importance food source for young larvae (Walker et al. 1991). Bioassay one moved liver powder solution into larval rearing containers when first instar larvae were added. Bioassay two removed the introduction of liver powder solution. Although the difference in the bioassay did not affect survival, it did have a significant effect on development rate. Bacteria associated with the environment of young larval instars are an important food source for young larvae (Lounibos et al. 1993). In treatments with liver powder solution added, it is possible that the additional nutrients provided cause larvae to develop significantly faster. However, future studies should look

to characterize the community of microorganisms within the larval rearing environment of mosquitoes. Other biological control strategies could look to manipulate the bacteria found in those environments because environmental bacteria often form transient symbioses with mosquitoes (Favia et al. 2007).

The recent releases of *Wolbachia* infected individuals into Australia have been deemed successful and may represent an advance in the control of disease vectors (Cyranoski 2012). However, *Wolbachia* affects insects in a variety of ways. Here, artificial *Wolbachia* infections decrease larval viability, which ultimately reduces the fitness of infected population. Infections that are associated with high costs are not predicted to invade populations, which limits the feasibility of population replacement. These same infections are good candidates for population suppression because they can induce high levels of CI, but are unlikely to cause accidental population replacement due to the high fitness costs. Case-specific studies should be designed to identify the effectiveness of *Wolbachia* as a vector population control tactic, but initial results are encouraging.

Table 2.1. Example life table for one replicate of the wild-type *Aedes polynesiensis* mosquito strain, APM. N_x is the number of individual adults alive in each age class at the beginning of the time period. D_x is the number of individuals that died during the time period. Mortality rate is the number of individuals that died in a given time period divided by the number of individuals that initialized the replicate. l_x is the survival of individuals in each age class. F_x is the fecundity of individuals with each age class. m_x is the per capita fecundity of individuals, and l_x m_x is the measure of reproduction and survival within each age class. R_0 is the net reproductive rate, i.e. the summation of all l_x m_x.

APM Replicate 3
Experiment 2

AGE INTERVAL (days)	N_X	D_X	*MORTALITY RATE*	l_x	F_X	m_X	$l_X m_X$
0-5	41	7	0.17	1.00	-	-	-
6-12	34	4	0.12	0.83	531.75	15.64	12.97
13-19	30	11	0.37	0.73	252.52	8.42	6.16
20-26	19	9	0.47	0.46	71.48	3.76	1.74
27-33	10	5	0.50	0.24	36.13	3.61	0.88
34-40	5	4	0.80	0.12	17.28	3.46	0.42
41-47	1	1	1.00	0.02	0.00	0.00	0.00
48-54	0	0	-	0.00	-	-	-
						R_0 =	21.75

Table 2.2. Summary of hatch rates from various crosses between different *Aedes polynesiensis* strains. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. In the table shown, the strain of the male is across the top, while the strain of the female is down the side. Some crosses are well-known from the literature and were thus only confirmed (*i.e.* two replicates confirmed qualitatively that crosses either produced viable or inviable embryos) by a C (compatible) or I (incompatible, see Brelsfoard et al. 2008, Brelsfoard and Dobson 2012). Novel crosses were replicated and analyzed statistically by a Mann-Whitney U-test. Means separation was determined by the Steel-Dwass all pairs nonparametric analysis, and crosses with different letters are statistically different ($p < 0.05$).

		Male		
		APM	MTB	CP
Female	APM	C	0.00%a	I
	MTB	0.00%a	75.50%b	0.00%a
	CP	I	77.78%b	C

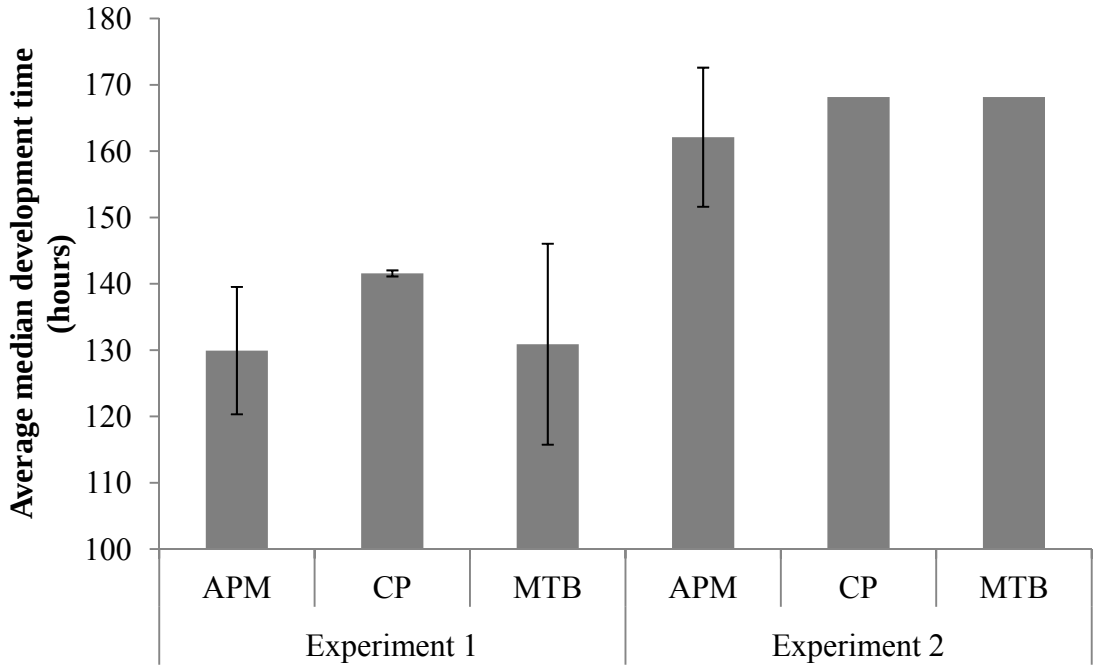


Figure 2.1. Average median development time for both experimental design one and two. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. There are no significant differences within experimental design, but mosquitoes developed significantly faster in experimental design one relative to two (Student's t-test; $p < 0.05$).

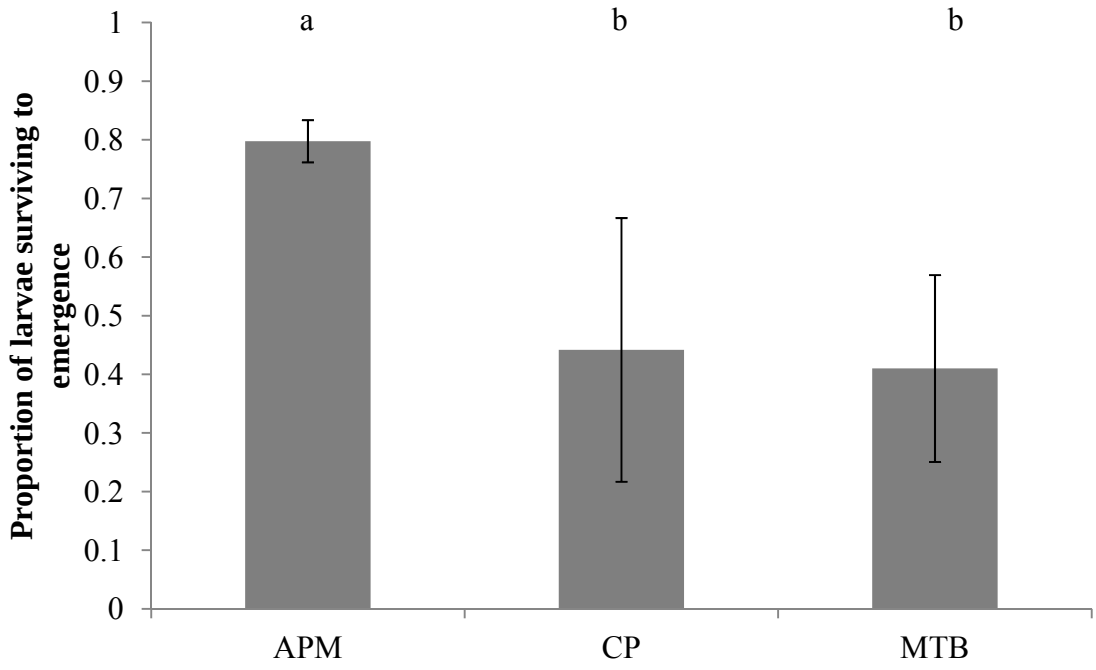


Figure 2.2.Proportion of larvae surviving to adult emergence for three strains of *Aedes polynesiensis*. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. Artificially infected strains (CP and MTB) had significantly lower survival than did the wild type strain. There was no difference between *Wolbachia* transfer techniques, *i.e*. introgression crosses or microinjection. Bars with the same letter are not significantly different (Tukey HSD; $p < 0.05$).

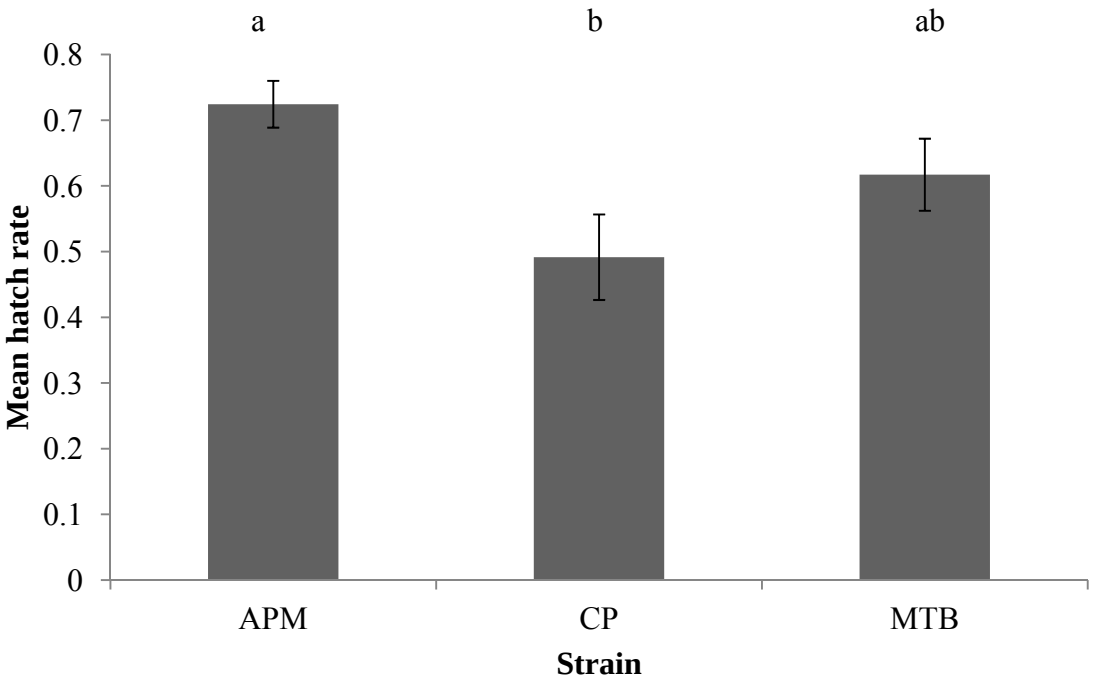


Figure 2.3. Mean hatch rate of eggs laid by three strains of *Aedes polynesiensis*. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. CP had significantly lower hatch rate than APM. MTB was intermediate and was equivalent to both APM and CP (Tukey HSD; = $p < 0.05$).

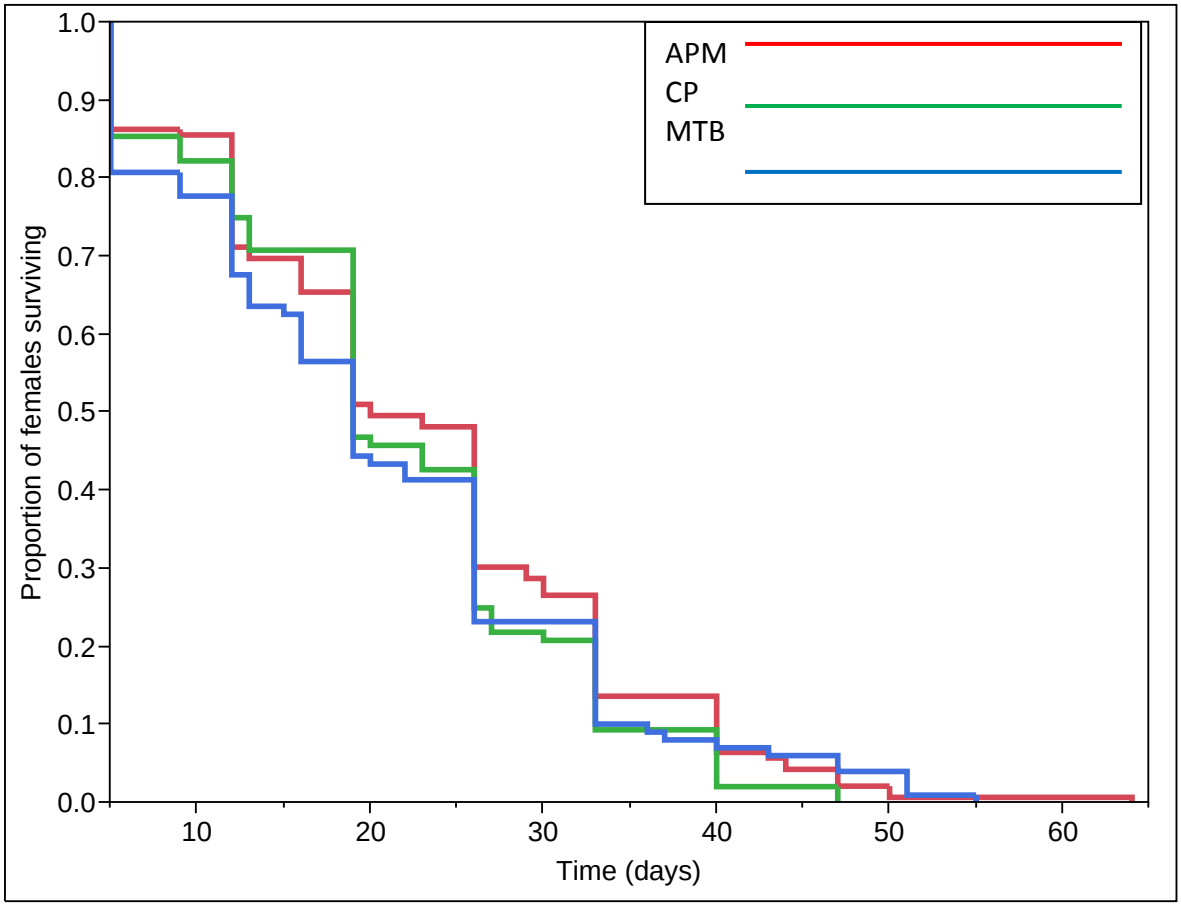


Figure 2.4. Survival curves for females of three strains of *Aedes polynesiensis*. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. There was no significant difference between strains for female survival (Cox-proportional hazard test; $p > 0.05$).

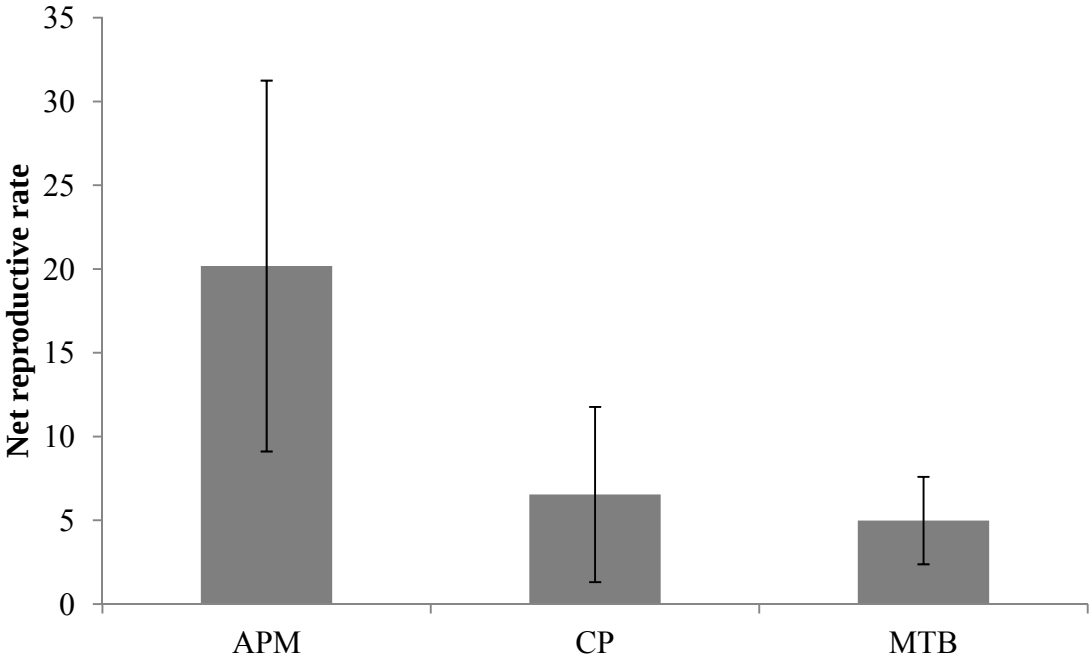


Figure 2.5. Estimates for net reproductive rate based on replicate life tables for three strains of *Aedes polynesiensis*. APM are wild-type individuals infected with *w*PolA. MTB is a mosquito strain which was artificially infected with *w*AlbB by microinjection. CP is a mosquito strain which was artificially infected with *w*RivB by interspecific introgression crosses. Bars with the same letter are not statistically different (Tukey HSD; $p < 0.05$).

Chapter Three

Wolbachia infections that reduce immature insect survival: Predicted impacts on population replacement

Introduction

The success of obligate endosymbiotic organisms depends on their ability to invade, establish and persist in their host. *Wolbachia pipientis*, a well-studied endosymbiont, is a species of maternally inherited bacteria in the order Rickettsiales, and infections are estimated to occur in more than half of all insect species (Hilgenboecker et al. 2008). Prior studies have demonstrated the ability of *Wolbachia* to manipulate the reproduction of its host (Werren 1997, Werren et al. 2008); several phenotypes have been described, including male-killing (Hurst et al. 1999, Hornett et al. 2009), feminization (Bouchon et al. 1998, Kobayashi and Telschow 2010), parthenogenesis (Huigens et al. 2000, Kremer et al. 2009, Stouthamer et al. 2010), and cytoplasmic incompatibility (CI) (Turelli and Hoffmann 1995, Dobson et al. 2002b, Farkas and Hinow 2010). CI affects a broad range of insect taxa and causes a reduction in egg hatch when *Wolbachia*-uninfected females and *Wolbachia*-infected males mate (Figure 3.1).

Prior models highlight three *Wolbachia*-specific parameters that affect the probability of *Wolbachia* invasion and establishment: the maternal inheritance rate, which is the proportion of infected offspring produced by an infected female; the level of CI, which is the proportion of embryos that fail to develop as a result of incompatible crosses (Engelstadter and Telschow 2009); and the fitness cost to females for carrying a *Wolbachia* infection, defined as a decrease in overall fecundity (Caspari and Watson

1959, Fine 1978, Hoffmann et al. 1990, Hurst 1991, Turelli and Hoffmann 1991, Turelli 1994).

Previous studies predict that the successful invasion of *Wolbachia* into an uninfected host population requires low fecundity costs, high maternal inheritance rates, and high levels of CI (Egas et al. 2002, Jansen et al. 2008). *Wolbachia* infections that impose a 10% relative fecundity cost to adult females experience reductions in their invasion success (Egas et al. 2002). Similarly, low maternal inheritance reduces the probability of *Wolbachia* invasion (Jansen et al. 2008). Higher initial *Wolbachia* infection frequencies are predicted to increase the probability of population replacement, which can offset the above costs (Engelstadter and Telschow 2009). Models have also addressed population structure at the adult stage, impacts on adult survival, stochastic effects, and overlapping generations (Egas et al. 2002, Rasgon and Scott 2004, Jansen et al. 2008, Engelstadter and Telschow 2009, Haygood and Turelli 2009, Turelli 2010).

The relative importance of *Wolbachia* effects on immature life stages has not been assessed theoretically. This is despite multiple examples demonstrating an effect of *Wolbachia* on immature hosts. In the stored product pest *Liposcelis tricolor* (Psocoptera: Liposcelidae), *Wolbachia* infections can decrease development periods and increase survivorship in some immature life stages (Dong et al. 2007). Other studies demonstrate negative impacts of *Wolbachia* infections on larval survival and development time (Islam and Dobson 2006, McMeniman and O'Neill 2010). Recent studies have determined that when intraspecific competition is intense, *Wolbachia*-infected mosquito larvae experience reduced survival (Gavotte et al. 2009, Gavotte et al. 2010).

To better understand population replacement by CI-inducing *Wolbachia*, both *Wolbachia* infection dynamics and host population dynamics were evaluated using a model that includes deterministic immature and adult male lifestages and a stochastic adult female lifestage. Since *Wolbachia* are transmitted maternally, the sex and infection status of hosts are explicit, and adult females are tracked individually. The focus of this modeling approach was to investigate changes in the probability of population replacement resulting from varying the relative larval viability (*RLV*), expressed as relative survival of infected to uninfected larvae. The results are presented in context with traditional parameters: the rate of CI, maternal inheritance (*MI*), the relative fecundity of infected females (*RF*), and the initial *Wolbachia* infection frequency (*IF*), on the probability of population replacement.

Methods

The model simulates a panmictic population that is closed to immigrants and emigrants. Consistent with previous studies, the model assumes mating is random and that *Wolbachia* infection has no effect on mating success. Females in the model mate once immediately upon reaching maturity. Adult survival is density-independent, but larval survival is density-dependent. The model presented here combines a stochastic adult female stage with deterministic adult male and immature stages. By implementing a deterministic immature stage, additional information regarding population dynamics is incorporated without developing a completely stochastic model, which would be considerably more computationally-intensive. The model incorporates overlapping generations (Turelli 2010) while tracking major life stages and considers females and

males separately. Development time and survival during immature stages are addressed explicitly by the model. The model was designed assuming the host is a holometabolous insect, and the model was parameterized based upon estimates of mosquitoes in the genus *Aedes* as a case study.

Brief Description of Equations

The following is a brief overview of all equations and parameters implemented in the model presented here. Additional development details, initial parameter values, and sensitivity analysis are provided in the Appendix.

$$R = \frac{(j-h)\Delta t e^{-qB} + h\Delta t}{s} \tag{3.1}$$

Larval development rate R (developmental stage units): j is the maximum development rate (developmental stage units), h is the asymptotic minimum development rate (developmental stage units), Δt is the time step (units of time), q is the density-dependent development coefficient (units of $(\text{mass})^{-1}$), B is the total larval biomass (units of mass) and s is the total number of developmental stages. Derived from Gavotte et al. (2009) and comparable to previously published data (Barbosa et al. 1972, Peters and Barbosa 1977).

$$S_L = e^{-(\mu+\alpha B^{\beta}+\gamma d^{-\varepsilon})\Delta t} \tag{3.2}$$

Larval survival, S_L: μ is the baseline mortality rate of mosquito larvae in the absence of competition (units of $(\text{time})^{-1}$). α is the coefficient controlling density dependent mortality (units of $(\text{time})^{-1}$). B is the total larval biomass (dimensionless), β is the exponent

controlling density dependent mortality (dimensionless), γ is the coefficient that decreases mortality as development stage increases (units of $(time)^{-1}$), d is the developmental stage index, ε is the exponent that decreases mortality as development stage increases (dimensionless), and Δt which is the time step (units of time). Based on Dye (Dye 1984) and similar to previously published studies (Southwood et al. 1972, Focks et al. 1993b, Magori et al. 2009).

$$M = \frac{m_x e^{k(d-1)}}{1 + \frac{1-c}{c}^{\frac{T_0 - T}{T_0}}} \tag{3.3}$$

Mosquito body mass, M (units of mass): m_x (units of mass) is the theoretical maximum mass of a given mosquito at time T. m_x is linked to c (dimensionless), which is the percent of m_x that is attainable. k (dimensionless) is the growth coefficient; T_0 (dimensionless) is the development time at which mass at pupation is $m_x/2$ days, and T (dimensionless) is development time. d (dimensionless) represents the total number of development stages completed by the larval cohort. Derived from previously published data (Gavotte et al. 2009).

$$F_s = e^{-gA} \tag{3.4}$$

Female survivorship, F_s: g is the per capita mortality rate of adult females (units of $(time)^{-1}$) and A is the current age of the female (units of time). Taken from Trpis and Hausermann (Trpis and Hausermann 1986).

$$E = u\Delta t e^{v(M_f + w)^z} \tag{3.5}$$

Egg production, E: u is the egg production rate; Δt is the time step (units of time); v is the female mass coefficient (units of $(\text{mass})^{-1}$); M_f is the body mass of the ovipositing female (units of mass); w is the female mass intercept (units of mass), and z is the female mass exponent (dimensionless). Derived by combining two previously published functions (Lounibos et al. 1985, Blackmore and Lord 2000).

Immature Life Stages

To simulate variation in egg hatch, the model assumes that some eggs (proportion equal to H_3, Table S1 in the Appendix) hatch on day three while the remaining eggs (1-H_3) hatch on day four (Figure 3.2a) (Christophers 1960, Gillett et al. 1977). Eggs are separated into two cohorts based on infection status. Larvae are distributed into four categories for each of the possible combinations of sex and infection status.

Larvae develop through discrete developmental stages, where the development rate is affected by density dependence, and larval survival is subject to both stage-dependent mortality and density-dependence (Figure 3.2b). The term "stage" is defined here as a measure of progress through larval development. The number of these discrete developmental stages is chosen to allow for variation in development time and is otherwise arbitrary (i.e., not linked to age or developmental instar explicitly). The number of larval developmental stages, s, can be varied, but was set to s=30 for this study. Larval development rate, R, is the number of developmental stages through which a cohort of larvae will pass within 24 hours (Equation 3.1). The number of larvae surviving to the next day is the product of the number of larvae in the preceding time period and the larval survival rate (Equation 3.2). When the number of developmental stages within a day is

not an integer, the larval cohort is distributed into two adjacent developmental stages in proportions that preserve the average development rate. The latter also introduces variation into the development rates of larval cohorts (Figure 3.2b). Density-dependence is based on the total mass of larvae (Equation 3.3). Male and female cohorts are considered separately to observe sex-specific patterns during development. For example, female mosquitoes require longer development time to become adults relative to males, and studies demonstrate that males and females respond to competition intensities differently (Gavotte et al. 2009).

Uninfected larval cohorts progress through development subject to stage-dependent mortality and density dependent effects only. Infected larval cohorts are subject also to a reduction in viability associated with *Wolbachia* infection. The relative larval viability (*RLV*, Table 3.1) for infected larvae is a proportion that indicates the relative survival of infected to uninfected larvae.

Following the completion of larval development stages, individuals become non-feeding pupae, which have a daily survival that is independent of population density (S_p, Table S1; Figure 3.2c). After completing pupal development, emerging male adults are tracked separately as either infected or uninfected cohorts. Emerging female adults are tracked as individuals.

Adult Life Stages

Six variables are tracked over time and determine the state of individual females: the blood meal state (time since last feeding), age (days since emerging), *Wolbachia* infection status (infected or uninfected), the *Wolbachia* infection status of her mate

(determined randomly based on the proportion of infected males in the population at the time she mates), size (body mass), and reproductive state (the number of gonotrophic cycles completed).

The probability that a female obtains a blood meal is determined by the frequency of potential blood meals per unit area, and each blood meal is associated with an additional mortality risk, regardless of mosquito age (Table S1). In the panmictic population simulated here, the availability of potential blood meals is assumed to be constant, but the model will allow downstream population structuring and geographic variation of bloodmeal availability.

Adult female daily survivorship F_s is age-dependent and probabilistic (Equation 3.4) (Trpis and Hausermann 1986). A female that is *Wolbachia* uninfected and mated with an infected male will lay eggs, but a proportion of the eggs will not hatch, depending on the level of CI (Table 3.1). Infected females produce viable offspring regardless of their mate's infection status but are subject to a decrease in relative fecundity (*RF*, Table 1). The number of eggs laid by an individual female is determined by her mass (Equation 3.5), and larval development influences female body mass. Specifically, intense competition delays development and reduces the mass of adult females.

Adult males, which are dead end hosts for *Wolbachia*, are not tracked individually but are tracked as infected and uninfected cohorts. The male mortality rate is assumed to be age-independent and constant (S_M, Table S1). The proportion of *Wolbachia* infected males in the population determines the probability of an incompatible mating for uninfected females.

Simulations

The model was written in MATLAB 7 (The MathWorks Inc., Natick, MA). A single simulation of the model produced population dynamics that are tracked over time (Figure 3.3). A series of simulations (n = 1000) were used to assess the impact of incremental parameter changes on the probability of population replacement. The parameters emphasized were cytoplasmic incompatibility (*CI*); maternal inheritance (*MI*); the relative fecundity of adult females (*RF*); the initial *Wolbachia* infection frequency, expressed as a proportion of the total number of adults (*IF*), and the relative larval viability (*RLV*). A population replacement event is defined as having occurred when the proportion of infected adults stabilizes above or equal to the *MI* value. During each series of simulations, individual parameters were varied singly, while the remaining parameter values were held constant as defined in Table 3.1. Each parameter was uniformly varied at one one-hundredth intervals from zero to one. At each interval, 1000 simulations were conducted, and the number of successful invasions was recorded to determine the probability of population replacement at that specific parameter value. The uniform sensitivity analysis was implemented for direct comparisons between all parameters across all intervals. Furthermore, previous analyses have not established minimum values for the spread of *Wolbachia*. Additional simulations tested two-way interactions between each of the emphasized parameters by varying two parameters simultaneously and evaluating the probability of population replacement. In the aforementioned simulations, parameters were varied uniformly. One parameter would be held constant while the other parameter varied as described above. The first parameter would then be incremented and the process above would be repeated. The probabilities resulting from two-way

interactions were approximately the product of the two parameters and are not discussed further.

Results

Figure 3.3 provides an example of the typical population dynamics resulting from model simulations of a *Wolbachia* population replacement event. In the illustrated example, the population begins as cohort of uninfected eggs and stabilizes after approximately 150 days, with variation around a consistent population size and lifestage distribution (Figure 3.3a). In the example simulation, the introduction of *Wolbachia* occurs at day 800 by introducing blood-fed, gravid adult females at an initial *Wolbachia* infection frequency (*IF*) of 0.5 (Table 3.1). *IF* is the frequency of *Wolbachia*-infected females relative to the total number of adults such that an $IF = 1$ is synonymous with a 1:1 (infected to uninfected) ratio. Figure 3.3b illustrates the resulting variation in *Wolbachia* infection frequency in the host population versus time.

Due to the stochastic nature of the model, the number of individuals within each lifestage fluctuates considerably over time (Figure 3.3a). To examine for temporal patterns in the fluctuations that might correspond to periodic signals such as stage durations or generation time, a spectral analysis was performed on the time series data for both total adult and larval populations via Fast Fourier Transformation (Wijnen et al. 2005, Keegan et al. 2007). The analysis can identify temporal patterns that exist in what appear to be chaotic time series. No pattern was detected by the spectral analysis. Since no period was found, stochasticity appears to be the sole driver of population fluctuations.

Five parameters associated with *Wolbachia* infection were evaluated for their affect on the probability of population replacement. The value of each parameter was varied at one one-hundredth increments, from zero to one, while additional parameters were held constant as defined in Table 3.1. For each parameter value, the probability of population replacement was determined by the number of successful replacement events occurring in 1000 simulations, for a total of 101,000 simulations per parameter.

Maternal inheritance (*MI*), the relative fecundity of adult females (*RF*), and relative larval viability (*RLV*), exhibit strong threshold behavior with population replacement occurring only at parameter values exceeding 0.7 (Figure 3.4). Specifically, realistic probabilities of population replacement (i.e., > 50% probability of population replacement) require the magnitude of *MI* to be greater than 0.9. Similarly, *RF* must exceed 0.9 before realistic probabilities of population replacement are attained. The probability of population replacement is most sensitive to *RLV*, which requires a value of greater than 0.95 before population replacement can occur. Furthermore, realistic probabilities of population replacement only occur at high *RLV* ($\geq$0.99), despite high maternal inheritance and CI (i.e., all other parameters held at values defined in Table 3.1).

A different functional relationship is observed with the level of incompatibility (*CI*) and initial *Wolbachia* infection frequency (*IF*), each of which results in response curves that increase asymptotically (Figure 3.4). Assuming the parameters within Table 3.1, the model predicts that CI is not necessary for *Wolbachia* to spread (i.e., approximately 7% of simulations resulted in population replacement when $CI = 0$). Realistic probabilities of population replacement occur when *CI* approaches 0.3. Despite

perfect CI (i.e., no egg hatch in incompatible crosses), population replacement did not occur in 10% of simulations (Figure 3.4). Additional simulations confirmed that a 90% probability of population replacement is an absolute maximum given the conditions defined here (Table 3.1). However, as the magnitude of *IF* increases, the probability of population replacement rapidly approaches one, with realistic probabilities of population replacement occurring when the frequency of infected females approaches 20% (Figure 3.4).

The results obtained from the model here were compared to a previously published stochastic model (Jansen et al. 2008). Table 3.2 compares the fixation probabilities calculated by the model presented here and those from Jansen *et al*. (2008) using the conditions defined in the prior report, which includes the introduction of a single infected female into a population size of 100 and perfect CI. To allow direct comparison, the relative larval viability in the presented model was set to one. 50,000 simulations were performed for each combination of parameter values used in the prior publication. Both models predict the probability of population replacement decreases when *MI* and *RF* values are less than one (Table 3.2). Generally, the predicted probabilities of population replacement were lower than the levels previously published. However, when either *MI* or *RF* was 80%, the model presented here reported higher probabilities (Table 3.2). Jansen *et al.* (2008) predicted that *Wolbachia* infections with imperfect maternal inheritance and low adult fitness costs (*MI* = *RF* = 0.9) will still invade and establish in a population, but no population replacement events were predicted by the new approach (Table 3.2). Similarly, the population replacement probability was determined assuming larger initial frequencies of *Wolbachia* infected

individuals (Figure 3.5). Both models predict an asymptotic increase in the probability of population replacement with increasing magnitude of *IF*, but Jansen *et al.* (2008) predicts higher probabilities of population replacement (Figure 3.5).

Discussion

The model presented here examines the probabilities of *Wolbachia* invasion into an isolated uninfected population. The model is unique in its individual-based representation of variation in key traits among adult females and in the resolution of larval dynamics within the host population. The model presented here predicts, as in previous modeling studies, that maternal inheritance (*MI)* and the relative fecundity of adult females (*RF*) are key parameters that determine the potential for population replacement. Specifically, population replacement occurs only at high *MI* or *RF*. In contrast, population replacement can occur at low *CI* or low *IF*. The simulation of adult females as individuals demonstrates that *MI* requires higher parameter values than *RF* for successful population replacement. The new parameter, relative larval viability (*RLV*), like *MI* and *RF*, requires high parameter values before population replacement can occur.

The relative larval viability between *Wolbachia* infected and uninfected individuals (*RLV*) is the most important determinant of population replacement, requiring the highest parameter values for invasion. The model predicts that reductions in infected larval survival can substantially reduce the probability of population replacement (Figure 3.4). While a majority of prior studies have examined for an effect in adults, recent studies have determined that, at high levels of intraspecific competition, *Wolbachia* infected larvae experience reduced survival (Gavotte et al. 2010). However, few

theoretical studies have examined the impact of immature lifestages on the invasion of *Wolbachia*. Here, it is demonstrated that reductions in *RLV* will inhibit *Wolbachia* invasion into an uninfected host population.

Recent work has highlighted the prevalence of *Wolbachia*, and its ability to invade populations (Turelli and Hoffmann 1991, Hilgenboecker et al. 2008). Studies have suggested that *Wolbachia* infection affects larval survival and development only when intraspecific competition is high (Hoffmann et al. 1996, Gavotte et al. 2010). Given the predictions discussed here, *Wolbachia* can only invade a population when *RLV* is very high. Therefore, the density of conspecifics in larval habitats is predicted to have significant impacts on the probability of population replacement. Similarly, the abundance and variety of larval habitats may have significant impact on the invasion of *Wolbachia*. The distribution, utilization and variety of larval habitats is well known for some insects, particularly mosquitoes (Harrington et al. 2005, Harrington et al. 2008, Koenraadt et al. 2008, Aldstadt et al. 2011). Theoretical studies considering the effect of metapopulation structure and larval rearing conditions may elucidate the mechanism by which *Wolbachia* can invade natural populations given low initial infection frequencies.

The level of CI in insects varies widely (Hoffmann et al. 1996, Charlat et al. 2003, Mercot and Charlat 2004, Zabalou et al. 2008). The model presented here shows that the intensity of CI has relatively little effect on the probability of population replacement when the rate of CI exceeds 60%. Furthermore, when $CI = 0$, population replacement can occur at low probabilities (Figure 3.4). Some *Wolbachia* infections do not cause CI, but are found at high frequencies in natural populations (Hoffmann et al. 1996, Hoffmann et al. 1998, Charlat et al. 2003). Previous theoretical studies indicate that CI or a sex-ratio

distorter is not required for population replacement when endosymbionts can alter female traits (Hoffmann et al. 1996, Hayashi et al. 2007). However, results presented here suggest that non-CI inducing *Wolbachia* infections can establish and persist in a population without increasing or altering host fitness, given high *MI*, *RF*, and *RLV*. Since the population considered by the model presented here is relatively small ($N \approx 110$ adults), genetic drift could perhaps influence the probability of population replacement (Hedrick 2011). To investigate the importance of genetic drift, the population size in the model was increased. In model simulations where the total adult population size is greater than approximately 200, population replacement does not occur when there is no effect of CI (i.e. $CI = 0$). However, when population size is increased, the general response patterns in Figure 3.4 are not altered.

High maternal inheritance rates have been observed consistently in natural populations (Poinsot et al. 2000, Rasgon and Scott 2003, Narita et al. 2007). Furthermore, theoretical studies predict the probability of population replacement declines as maternal inheritance decreases (Egas et al. 2002, Jansen et al. 2008, Farkas and Hinow 2010). Similar to previous studies, results presented here suggest that maternal inheritance (*MI*) must be high for a *Wolbachia* infection to invade an uninfected population and persist. Specifically, *MI* must be higher than 90% to attain a realistic probability of population replacement.

The effect of *Wolbachia* infections on adult female fitness has been well documented empirically and theoretically (Caspari and Watson 1959, Fine 1978, Turelli and Hoffmann 1995, Weeks et al. 2002, Weeks et al. 2007, Jansen et al. 2008, Turelli

2010). Here, as in previous theoretical studies, the model predicts that the relative fecundity of adult females (*RF*) must be high to facilitate population replacement.

For all parameters, the probability of population replacement approached an absolute maximum of 90% given the conditions defined in Table 3.1. Here, the initially examined *IF* value is relatively high (0.5), analogous to artificial introductions examined in prior theoretical work (Rasgon and Scott 2004). Subsequently, lower *IF* values have been simulated (Figure 3.4), including the introduction of a single, infected female (Table 3.2). The model predicts that *Wolbachia* invasion can occur at the lowest *IF* values and demonstrates an increasing probability of invasion with the higher introduction levels, with the probability of population replacement approaching 100%. Additional simulations determined that when *IF* is held constant and the total adult population size is increased, the probability of population replacement approaches one given the conditions defined in Table 3.1. This result suggests genetic drift can affect the probability of population replacement in small populations and may facilitate or hinder the spread of *Wolbachia* from low initial frequencies (Hedrick 2011).

The model presented here predicted lower population replacement probabilities than those predicted by previous stochastic models (Table 3.2 and Figure 3.5) (Jansen et al. 2008). Rasgon and Scott (2004) noted a similar behavior where implementing population age-structure and overlapping generations increased deterministic thresholds. The inclusion of additional life stages and stage-structure in this stochastic model may explain the reduced probabilities of population replacement. However, the model presented here predicted marginally higher probabilities of population replacement when either maternal inheritance or the relative fecundity of infected females had a magnitude

of 0.8. The increased probability of population replacement predicted by the model presented here is likely a result of the individual-based representation of the adult female life stage that includes stochastic survival.

The model here addresses a single, panmictic, isolated population but could be expanded to include metapopulation structure. If introduction events can be assumed to occur randomly, then the surrounding subpopulations should generally tend to inhibit population replacement, because migration between subpopulations would dilute the proportion of infected individuals. However, as demonstrated here, genetic drift may influence the invasion of *Wolbachia* in smaller subpopulations. The spatial spread of *Wolbachia* has been assessed analytically by others and defines the conditions needed for *Wolbachia* to spread through space (Turelli and Hoffmann 1991, Turelli 2010).

The majority of models that address the invasion of *Wolbachia* into uninfected populations have examined populations without lifestage subdivisions, suggesting that additional empirical studies focused on understanding larval dynamics are needed (Magori et al. 2009). Many of the parameters defined here may be difficult to determine in natural populations (Rasgon and Scott 2004), but the results demonstrate the importance of understanding the role of life history parameters and their interactions, despite the difficulties. Furthermore, the sensitivity analysis of the model presented here demonstrates that the magnitudes of particular parameters strongly influence the potential for spread and establishment of *Wolbachia*; these (e.g., *Wolbachia* effects on immature fitness) should be the focus of future empirical and theoretical studies. Future theoretical studies could further address parameter sensitivity by hyper-cube sampling, but this

would require information about the distribution of parameters to investigated (Kiparissides et al. 2009).

Wolbachia is currently being utilized as the basis for a gene drive strategy in open field releases of *Aedes aegypti* (Marshall 2009, Enserink 2010); however, the predictions of the model presented here suggest that minute reductions in *RLV* can inhibit population replacement. Research needs to focus on understanding the effects of novel *Wolbachia* infections on immature lifestages. Xi *et al*. (Xi et al. 2005a) demonstrated that novel *Wolbachia* infections can establish in a new host species and replace an uninfected population, but the initial frequency of *Wolbachia* infected individuals needed to replace the population was higher than predicted. The authors suggested that differences in survival of immature lifestages could explain their results. Results presented here indicate that even reductions in *RLV* that are difficult to detect empirically will substantially reduce the probability of population replacement.

The rapid decline in the probability of population replacement associated with reduced larval viability indicates that empirical studies directed toward quantifying the effects of endosymbionts on immature insects are important for understanding and predicting *Wolbachia* invasion events. Recent empirical studies also suggest that a more complete understanding of the effects of *Wolbachia* on the immature life stages is generally needed through additional empirical and theoretical studies (Gavotte et al. 2009, Gavotte et al. 2010, McMeniman and O'Neill 2010).

Table 3.1. Glossary of notation, including the initial values for each key parameter. In all subsequent model runs, each value remains constant while one key parameter is varied. (For a list of all population dynamic parameters, see Table S1 in the Appendix.)

symbol	definition	initial value
CI	proportion of embryos not hatching in incompatible CI crosses	0.999
MI	proportion of offspring receiving infection (maternal inheritance)	0.999
RF	relative fecundity of infected females to uninfected females	0.999
RLV	relative larval viability of infected larvae to uninfected larvae	0.999
IF	initial frequency of gravid infected females to the total adult population	0.500

Table 3.2. The probability of population replacement for given parameter values. The probability of population replacement for given parameter values, assuming perfect CI and the release of a single infected adult female into an uninfected population with a size of 100. The fixation probabilities from Jansen *et al.* (2008) are generally higher than model predictions, except when maternal inheritance and the relative fecundity of infected adults is 0.8. The model presented here predicted population replacement would not occur when both maternal inheritance and the relative fecundity of infected adult females were 0.9. All probabilities generated from the model presented here reflect the proportion of population replacement events that occurred per 50,000 simulations of the model. Comparable values were estimated from Figure 2 in Jansen *et al*. (2008).

MI	*RF*		
	1.0	0.9	0.8
1.0	0.1023 / 0.0359	0.0224 / 0.0089	0.0004 / 0.0007
0.9	0.0158 / 0.0060	0.0004 / 0.0000	/
0.8	0.0001 / 0.0004	/	/

Jansen *et al*. (2008) / model presented here

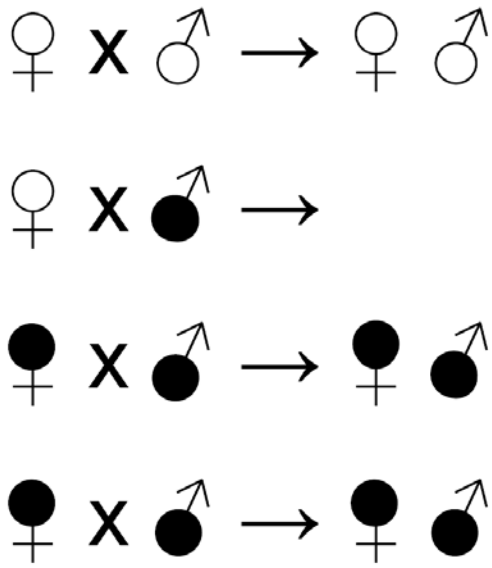


Figure 3.1. Unidirectional cytoplasmic incompatibility crossing pattern.

White circles represent uninfected individuals and black circles represent *Wolbachia* infected individuals. Crosses between the same infection type produces viable offspring. Cytoplasmic incompatibility occurs when uninfected females mate with *Wolbachia* infected males, resulting in reduced numbers of viable offspring. As a result, infected females have an effective mating advantage over uninfected females.

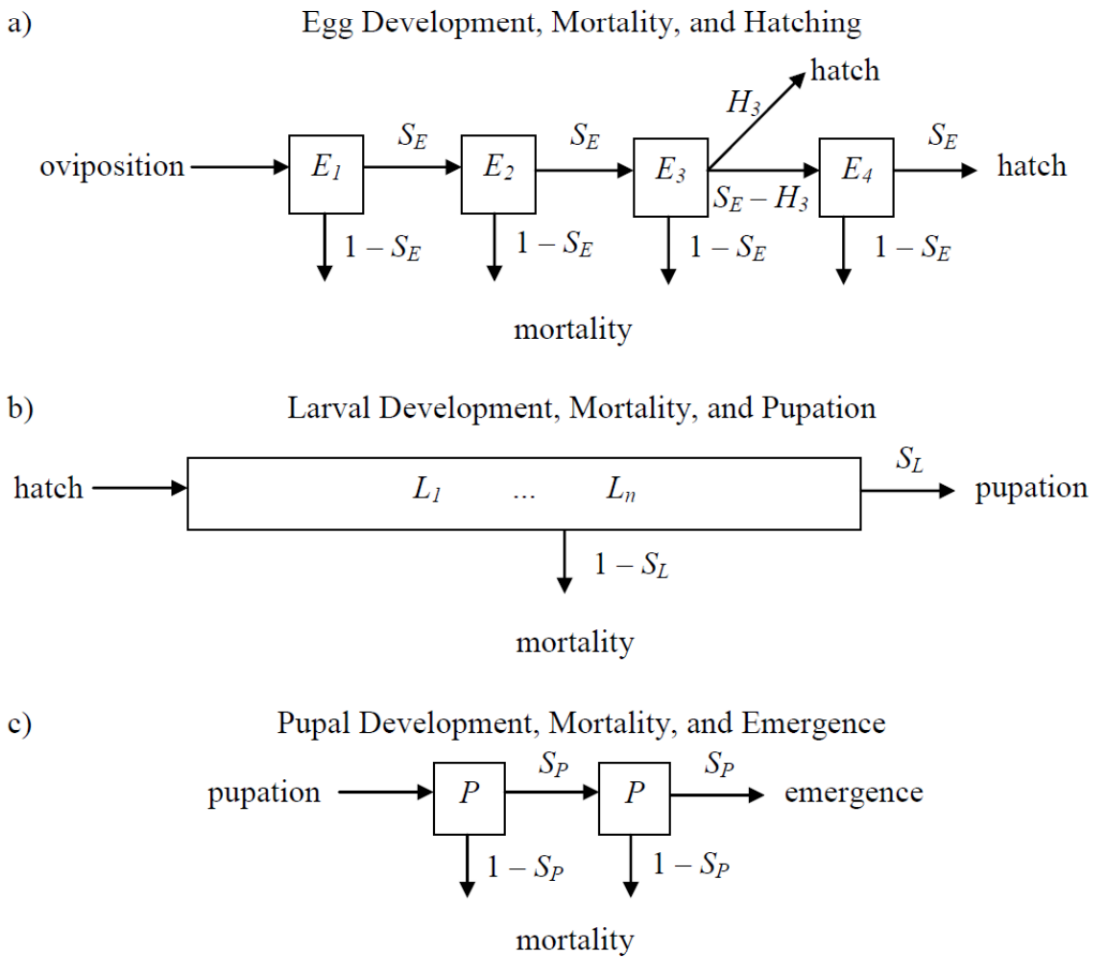


Figure 3.2. Immature population structure. a) Eggs develop through four discrete stages and each stage is one day. There are two cohorts of eggs, *Wolbachia* uninfected and infected. During development, eggs move through each stage consecutively, and the number of eggs advancing to the next stage reflects the product of the number of eggs present and S_E, daily egg survivorship (Table S1). All eggs hatch after four days except a proportion of eggs hatch at day three (H_3, Table S1). b) Larvae develop through s discrete stages, where s is an arbitrary number of developmental stages (s = 30). Larvae are divided into four categories: *Wolbachia* infected/uninfected and male/female. Larvae move R developmental stages in each time step, where R is the number of developmental

stages a larval cohort will progress (Equation 3.1). The number of larvae progressing from their current development stage, e.g. L_2, to their next developmental stage, L_{2+R}, is equal to the product of the number of larvae in a developmental stage and larval survival (Equation 3.2). Larval survival and development are density dependent. If larvae are *Wolbachia* infected, they are subject also to the parameter RLV (Table 3.1), which can reduce the number of surviving larvae. Larvae that reach the last developmental stage become pupae. c) Pupae progress through two discrete development stages and are tracked similar to eggs. Each pupal developmental stage is one day and pupae are subject to S_P, daily pupal survivorship (Table S1).

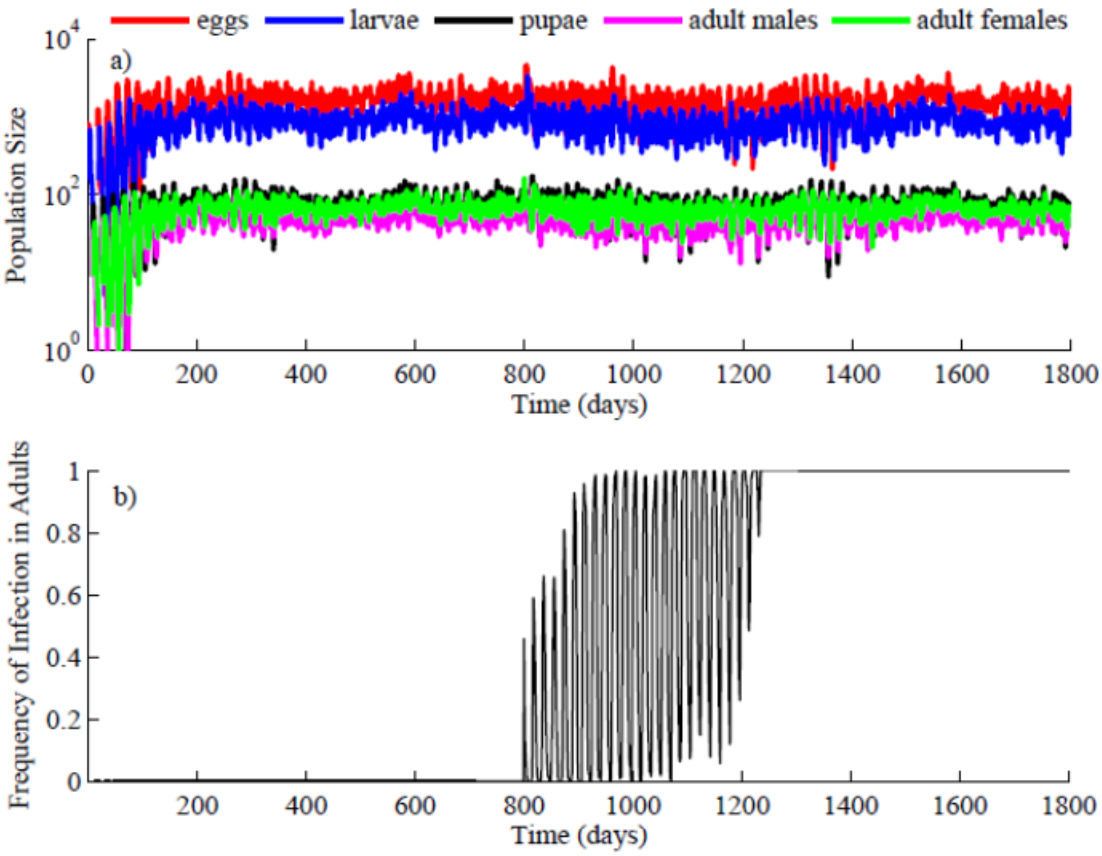


Figure 3.3. Example of typical population dynamics produced by a simulation of the model. a) Populations begin with an uninfected cohort of eggs. The population is allowed to persist and self-regulate for 800 days, at which time *Wolbachia* is introduced to the population as gravid, bloodfed females at the rate defined in Table 3.1. The population is then allowed to self-regulate and persist until 1800 days have elapsed. b) The proportion of the female population that is infected with *Wolbachia* over time (i.e., infection frequency), demonstrating a population replacement event.

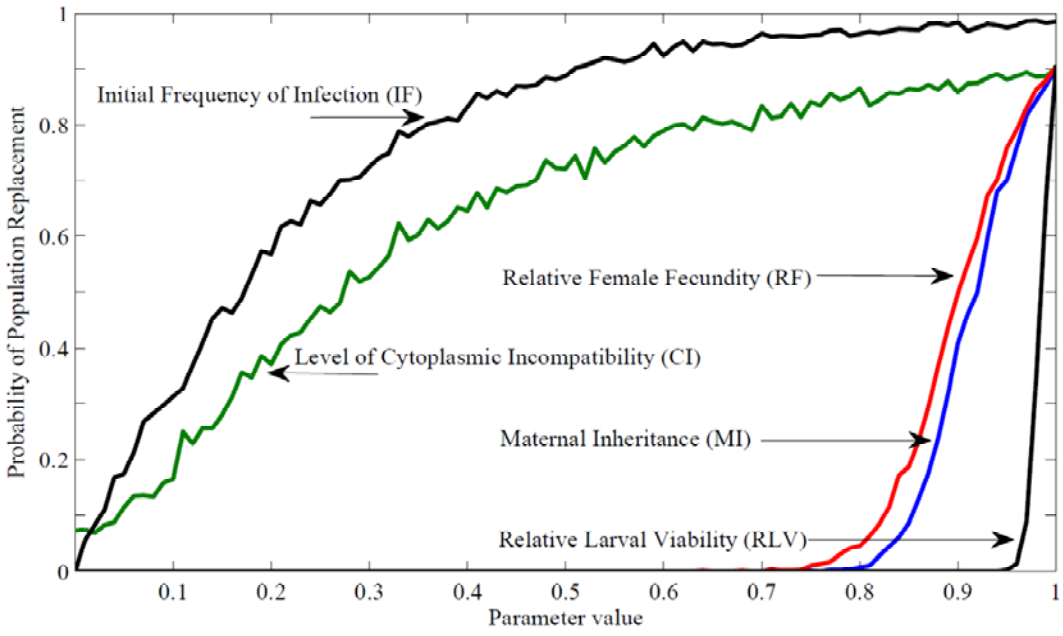


Figure 3.4. The probability of population replacement for five *Wolbachia* specific parameters. *CI* is the level of cytoplasmic incompatibility, *MI* is the level of maternal inheritance, *IF* is the initial frequency of *Wolbachia* infection, *RF* is the relative fecundity of *Wolbachia*-infected adult females, and *RLV* is the relative larval viability. Each line was generated by calculating the probability of a population replacement event at one one-hundredth increments for parameter values between zero and one (n=1000 simulations/increment). *IF* and *CI* show similar responses to parameter value increases. The probability of population replacement increases, but then asymptotically approaches one. The response curves for *RF*, *MI*, and *RLV* behave similarly, each parameter requiring values to be greater than approximately 0.7. The curves then quickly increase toward one. *RLV* is the most sensitive parameter requiring values approaching 0.95 before a population replacement event can occur.

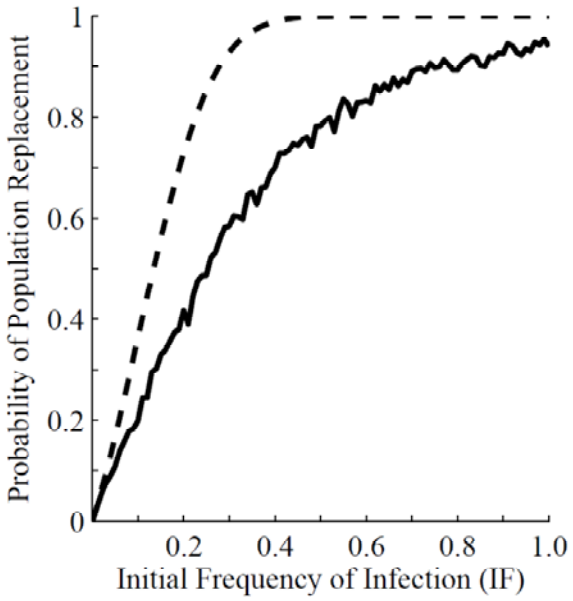


Figure 3.5. The probability of population replacement by *Wolbachia* given different initial infection frequencies. This figure assumes that the relative fecundity of infected females is 0.95, with perfect CI and maternal inheritance. The dashed line indicates the probability of population replacement as calculated by Jansen *et al* (2008), and the solid line represents the predictions of this model. The functional response here is similar to the prior publication, but predicts lower probabilities at all initial *Wolbachia* infection frequencies.

Chapter Four

Wolbachia re-Replacement without Incompatibility: Potential for Intended and Unintended Consequences

Introduction

Estimates are that 2.5 billion people are at risk of dengue in 100 endemic countries. Fifty million new infections annually result in more than 22,000 deaths, primarily among children (Gubler 2002, Kyle and Harris 2008, Guzman et al. 2010). Infection with one of the four Dengue virus (DENV) serotypes in humans can result in multiple syndromes, ranging from subclinical to mild febrile infections to death. The more severe syndromes can occur upon secondary dengue infection with a different DENV serotype, through a process known as immune enhancement (Guzman et al. 2010). The probability of the latter has increased in recent decades with the spread of dengue, and currently all four dengue serotypes co-circulate in Asia, Africa and the Americas (Rodriguez-Roche et al. 2005). Substantial effort is being devoted to developing vaccines and antivirals against dengue, but efforts are complicated by the risk of vaccine-induced immune enhancement. There are encouraging results in recent trials, with several candidate vaccines in development and with one candidate in Phase 3 trials (Guy et al. 2011, Schmitz et al. 2011). At present however, mosquito control and avoidance remain the only tools against dengue.

While additional mosquitoes (e.g., *Aedes albopictus*) are important dengue vectors, *Aedes aegypti* is the major urban vector (Jansen and Beebe 2010). Originating in Africa, *Ae. aegypti* is thought to have begun its global spread in the fifteenth century

(Christophers 1960), but a recent acceleration of its expansion has resulted from global transportation and urbanization (see review (Jansen and Beebe 2010)). The importance of *Ae. aegypti* as a vector is due in part to its close association with humans and a preference for human hosts. Adult *Ae. aegypti* commonly rest within buildings and breed frequently in artificial containers near homes. For brevity, its importance as a dengue vector is highlighted here, but *Ae. aegypti* is an important vector of other pathogens also, including Yellow Fever (from which it derives its common name, the Yellow Fever mosquito), chikungunya and additional pathogens important to humans, livestock and wildlife (see review (Christophers 1960)).

As a day-active mosquito, bed nets and similar protections of resting persons are not effective. Instead, *Ae. aegypti* control relies heavily upon source reduction and insecticides (World Health Organization 2009). Source reduction/environmental management to reduce the number of breeding sites is a recommended and proven nonchemical method for control, but it requires sustained community participation (Heintze et al. 2007, Ballenger-Browning and Elder 2009). Larviciding against immature mosquitoes is used commonly but can miss many breeding sites that are cryptic or inaccessible, and its efficacy at reducing dengue transmission risk is a subject of discussion (Scott and Morrison 2004, Morrison et al. 2008, Barrera et al. 2011). Vehicle-mounted adulticiding can have limited efficacy against *Ae. aegypti*, since these treatments can fail to reach adults that rest indoors (Castle et al. 1999, Perich et al. 2000). Common alternative adulticides are indoor space spraying and residual spraying, using chemicals with low mammalian toxicity. However, the efficacy of chemical-based controls is inhibited by insecticidal resistance. Most control programs rely upon two of the four

insecticide classes, resulting in increased selection pressure on mosquito populations to develop resistance. With identified resistance to all four insecticidal classes in *Ae. aegypti*, there has been substantial investment to develop new chemicals, but estimates are that it will take at least a decade before the new active ingredients become available for mosquito control (Ranson et al. 2010). In the interim, recommendations for *Ae. aegypti* control include relying on nonchemical control whenever possible (Ranson et al. 2010).

A tool being developed and field-tested is based upon infections of *Wolbachia* pipientis, which is a maternally inherited, obligate intracellular bacterium estimated to occur naturally in approximately half of all insect species (Hilgenboecker et al. 2008). The evolutionary success of *Wolbachia* is attributed in part to its ability to manipulate the reproduction of its host to promote infection. *Wolbachia* occurs naturally in Aedes and Culex mosquitoes where it can induce a form of conditional sterility known as cytoplasmic incompatibility (CI). While the underlying molecular mechanism of CI is unknown, it has been described as modification-rescue: *Wolbachia* present in the male modify the sperm such that karyogamy failure will occur unless the female carries a similar *Wolbachia* type, which rescues the modification (Charlat et al. 2001). Unidirectional CI occurs within host populations that include both infected and uninfected individuals. The rescuing *Wolbachia*-infected females are compatible with all males in the population; however, uninfected females produce progeny only when mated with non-modifying, uninfected males. The resulting reproductive advantage to *Wolbachia*-infected females can drive the spread of *Wolbachia* into an uninfected

population, and the latter has been described in naturally-occurring population replacement events (Turelli and Hoffmann 1991, Weeks et al. 2007).

With an ability to artificially generate *Wolbachia* infections in mosquitoes (Xi et al. 2005b, Fu et al. 2010), applied public health strategies have rapidly advanced in recent years, and field trials are ongoing in Australia (Hoffmann et al. 2011). Important to the strategy is the ability of *Wolbachia* to interfere with dengue replication, i.e. *Wolbachia* is associated with a reduction in dengue virus titers, as demonstrated in laboratory assays (Walker et al. 2011). Results from the field trial are encouraging in that repeated releases of *Wolbachia* infected *Ae. aegypti* resulted in replacement of the naturally uninfected *Ae. aegypti* population with the *Wolbachia* infected cytotype in two Australian cities. The *w*Mel *Wolbachia* strain was used in this early trial.

A potential complication is that the level of dengue interference by the *w*Mel strain may not be as robust as originally believed (Cyranoski 2012). Thus, additional releases have been initiated with the *w*MelPop infection type in two additional Australian cities (Cyranoski 2012). Lab assays show the level of dengue interference caused by the *w*MelPop infection to be higher than that caused by wMel (Walker et al. 2011). Although the *w*MelPop infection was originally intended for the initial field releases, a decision was made to use the *w*Mel strain, since it had lower host fitness costs relative to the *w*MelPop strain (Walker et al. 2011).

Host fitness costs of *Wolbachia* infections can slow or prevent *Wolbachia* establishment and spread (Barton and Turelli 2011, Crain et al. 2011, Hancock et al. 2011). Directly related to the Australian public health strategy, models predict that *Wolbachia*-induced host fitness costs require relatively larger releases of *Wolbachia*

infected females to accomplish the goal of population replacement (Turelli 2010). Models predict that above a fitness cost threshold, *Wolbachia* cannot be sustained in populations without ongoing release of infected females (Barton and Turelli 2011). For field releases, it is important to release a minimal number of hematophagous females, due to the potential health effects to the inhabitants of the cities in which the releases occurred.

The recent establishment of both the *w*Mel and *w*MelPop infections in different Australian cities has created a novel situation, which has not been addressed in modeling analyses and is relevant to public health. Specifically, while prior models have examined interactions of *Wolbachia* with different modification-rescue types, models have not examined *Wolbachia* dynamics predicted to result from differing host fitness costs. Here, I describe a model of two *Wolbachia* infection types that are similar, with the exception that they differ in their effect on host fitness.

Methods

Theoretically, I consider two infection phenotypes. One infection type is commensal (the wC infection), which causes relatively little fitness cost to its host. The second infection is pathogenic (the wP infection) and causes a relatively high host fitness cost. Both infections cause strong unidirectional CI in crosses with uninfected hosts. However, I assume that mating between hosts infected with the *w*C and *w*P *Wolbachia* types is compatible, i.e. CI does not occur in reciprocal crosses between individuals infected with *w*C or wP.

The model on which our analysis is based has been described previously (Crain et al. 2011). In the previous publication, the model considers the introduction of one

Wolbachia infection into an uninfected population. Briefly, the model is a life history model that uses matrices to track immature development and survival for both sexes and for each infection type. All immature life stages are deterministic and the equations dictating development and survival are parameterized for a mosquito-specific system. Adult female mosquitoes are modeled individually with several associated state variables, and adult males mosquitoes are modeled deterministically. Populations are initiated with uninfected individuals and the release of different *Wolbachia* infections is simulated (see Crain et al. 2011).

To consider both *w*C and *w*P infections, the data structures in the model were replicated, but otherwise the model was unaltered. To differentiate infection phenotypes, I focus on a single parameter Δ, which is a fitness multiplier ($0 < \Delta < 1$): a measure of the mosquito fitness associated with a *w*P infection, relative to that of a *w*C infection. Lower Δ values equate to reduced larval viability (*RLV*), female fecundity and adult survival of hosts infected with *w*P infection type. To simplify simulations, the model assumes that only the larval stage is affected by Δ (*i.e.* egg and pupal survival remained high). For simulations described here, the *w*C infection parameters remain constant and are identical to those previously described (Crain et al. 2011). Note that the *w*C infection parameters were held constant, but additional simulations were conducted in which *w*C was associated with an increased fitness costs to its host. Since Δ is a relative multiplier, increasing cost to *w*C infections did not alter the general patterns described here, and therefore changes to the *w*C parameters are not discussed further.

Preliminary simulations determined the population replacement probability for either *w*P or *w*C infected individuals invading an uninfected population with the total

number of simulations held constant (n = 1000 simulations). For these simulations, the initial infection frequency was held at 0.5, and *RLV* was varied; Δ was held constant at 0.993.

To simulate two *Wolbachia* introduction events, the time duration for a single simulation was increased to 2600 days. The release of *w*P infected individuals was identical to that defined in the previous report (Crain et al. 2011). Following the initial release, populations were allowed to stabilize for 800 days. After population stabilization, a second release occurred at day 1600 where the entry ratio of *w*C individuals (*Π*) was set to 0.5. The entry ratio, *Π*, represents a proportional release of gravid *w*C females relative to the total adult population size such that *Π* = 1 is synonymous with a 1:1 release ratio. Following the second release, 1000 days were simulated to evaluate the ability of *w*C infections to invade *w*P populations. To determine the probabilities of *w*C invading a *w*P-infected population, the parameters *Π* and Δ were varied in tandem while all other parameters in the model were held constant, as defined previously (Crain et al. 2011). Specifically, I examined six values for *Π* and each 0.001 increment of Δ from 0.97 to 1.00. For each combination of parameter values, the probability of a *w*P invasion was calculated, and following successful *w*P invasion, the probability of population replacement by *w*C infections was calculated. For Δ values less than 0.985, the invasion of *w*P infections was very low, such that sufficient replacement did not occur in over 100,000 simulations, even at the highest introduction rate, *Π* = 1.

Additionally, simulations were designed to represent a hybrid-zone where *w*C and *w*P infections overlap in their distribution. In these zones, the infections are assumed to be present at equal proportions. Simulations started with a population infected with equal

proportions of *w*C and *w*P infected individuals and then infection levels were tracked for 1000 days. Similar to above, Δ was varied between 0.97 to 1.00, and I determined the probability that *w*C infections would become dominant. As Δ approached 1.00 (*i.e.*, the two infection types become equivalent) infections often did not reach fixation. To be classified as dominant, an infection must exceed a 50% infection frequency for the final 100 days of the simulation.

Results

Both *w*C and *w*P infections can invade uninfected host populations (Figure 1). Despite host fitness costs caused by *Wolbachia*, the infections can invade due to unidirectional CI, leading to populations that are stably infected with *Wolbachia* (Crain et al. 2011). Due to the higher host fitness costs associated with infection, *w*C infections have higher probabilities of population replacement relative to *w*P. However, since the latter was the focus of a prior publication (Crain et al. 2011), I do not discuss the latter point here. If the populations remain separate, then the dynamics are similar to that previously described. Here, I consider potential outcomes in the event that the populations are not completely isolated from each other.

In the initial simulations, I consider a scenario (Scenario I) in which females infected with *w*C enter into a population that is stably infected with the *w*P infection type (Figure 2). The mode of *w*C may be by release or by immigration. A single introduction event is modeled by releasing *w*C-infected mosquitoes into a population of *w*P-infected mosquitoes. Simulated populations consist of all life stages (i.e., adult males, adult females, pupae, larvae, and eggs). Thus, considering the entire population, adult females

infected with the immigrated *w*C infection type make up a minority of the total population, even when the number of entering adult females infected with *w*P is equal to the number of adult females infected with the established *w*C infection ($\Pi = 1.0$).

As shown in Figure 3, the *w*C infection can invade a population previously infected with *w*P, resulting in a population that is infected exclusively with the introduced *w*C infection type (*i.e*., population replacement). As expected, the probability of population replacement increases with the relative number of immigrating females (*i.e.*, higher entry ratio Π). If the two *Wolbachia* types are identical in host fitness ($\Delta = 1.0$), then the infection frequencies vary by drift only. Since the invading *w*C females make up a minority of the overall population at the start of all simulations shown in Figure 3a, there is essentially no chance of a successful *w*C invasion as Δ approaches one. With greater differences between the host fitness costs of the two infection types, *i.e*. at lower Δ values, the probability of replacement becomes high. Simulations of the opposite direction (*i.e*., introduction of a *w*P infection into a population that is stably infected with the *w*C infection type) show that *w*P is unlikely to invade *w*C. Specifically, the female entry rate must be at least twice the number of the *w*P population ($\Pi = 2.0$), which resulted in replacement of the *w*C population in <5% of simulations.

In a second set of simulations, I consider a scenario (Scenario II) in which the two populations overlap across at least part of their distribution (Figure 3b). Thus instead of the entry of adult females only, as above, simulations start with equal numbers of individuals infected with the different *Wolbachia* types. As shown in Figure 3b, there is a high probability of *w*C invasion across a broad range of Δ values. Therefore, replacement of the *w*P type by the *w*C infection is predicted even at small differences in

the host fitness effects caused by the two infection types. It is only when the two infections become essentially identical in their host fitness costs ($\Delta \approx 1.0$), that the probability drops to 0.5 and the infection frequencies change by drift only.

Discussion

The *w*MelPop infection has measurable negative effects on the fitness of infected *Ae. aegypti* (McMeniman et al. 2009, Suh et al. 2009, Yeap et al. 2011), making it an example of a pathogenic infection (*i.e.*, *w*P). In contrast, the effects of the *w*Mel infection on host fitness are relatively minor (Walker et al. 2011), placing it in the category of a commensal infection (*i.e.*, wC). The differences in host fitness effects were important in the decision to release *w*Mel instead of *w*MelPop into two Australian cities in 2010 (Hoffmann et al. 2011, Walker et al. 2011). In 2011, mosquitoes infected with *w*MelPop were released in two additional Australian cities (Cyranoski 2012) because of this strain's apparent reduction in dengue transmission by host mosquitoes. Ongoing reports describe high levels of all infections in each of the cities receiving released females (http://eliminatedengue.com).

Our models predict that if the *w*Mel and *w*MelPop infections do not remain geographically separated, there is the potential for the *w*Mel infection to invade and replace the *w*MelPop infection. Since the *w*MelPop infection is described as having more desirable traits for interrupting dengue transmission, its replacement by *w*Mel represents a potentially undesirable outcome. Specifically, this would replace a *Wolbachia* infection causing strong dengue interference with one causing reduced dengue interference.

How might the two infections achieve spatial overlap? *Ae. aegypti* is a container breeding species with embryos that can desiccate and still persist for months (Juliano et al. 2002). Its propensity for breeding in artificial containers (*e.g.*, flower pots, tires) has contributed to its global spread (Jansen and Beebe 2010). Thus, there is a possibility of human-assisted transport between cities in Northern Australia. This would be analogous to Scenario I described above (Figure 4). The cities in Northern Australia were selected in part due to surrounding areas that are inhospitable to *Ae. aegypti*, which serve as barriers against immigration/emigration (Endersby et al. 2011). However, infestation of these cities by *A. aegypti* demonstrates that their isolation is incomplete.

Both *w*Mel and *w*MelPop cause unidirectional CI in crosses with the indigenous *Ae. aegypti*, which are naturally uninfected with *Wolbachia*. Model predictions (Turelli 2010, Barton and Turelli 2011) as well as empirical trials within containment (Walker et al. 2011) demonstrate the ability of both *w*Mel and *w*MelPop to invade and replace uninfected populations. Thus, the geographic spread of one or both *Wolbachia* types into the surrounding indigenous, uninfected *Ae. aegypti* population may occur, similar to the Bartonian wave described during a *Wolbachia* infection in a California population of *Drosophila simulans* (Turelli and Hoffmann 1991). The range expansion of one or both infection types can lead to spatial overlap, analogous to Scenario II (Figure 4).

The CI pattern that results from crosses between *Ae. aegypti* infected with either *w*Mel or *w*MelPop is an important unknown that would affect model predictions. The infection types are similar, and in *D. melanogaster*, *w*MelPop females were able to rescue the *w*Mel modification in males (McGraw et al. 2002). However, I am unaware of similar crosses having been conducted in the *Wolbachia* transinfected *Ae. aegypti* strains.

Here, model simulations are based upon an assumption of reciprocal compatibility between *w*Mel and *w*MelPop. The predictions presented here encourage additional empirical tests within containment, including specific tests of hypothesized events associated with sympatry of *w*Mel and *w*MelPop. With more laboratory data on the extent of reciprocal compatibility, our model can be modified to predict responses in the field more accurately. Upon confirmation of the potential for replacement of *w*MelPop by *w*Mel, it may be possible and desirable to eliminate the *w*Mel infections from the currently restricted areas in which the infection occurs.

The dynamics occurring between infections that differ in their host fitness cost may be used advantageously, such as with unidirectional incompatibility applied for the repeated replacement of populations or reversal of replacement events (Dobson 2003). The model predictions described here show that the *w*Mel infection would serve as a vehicle for replacing/removing the *w*MelPop infection from a population. While in the *w*Mel/*w*MelPop example, this is a potential liability, the general strategy can be used advantageously in the design of additional *Wolbachia*-based applied interventions.

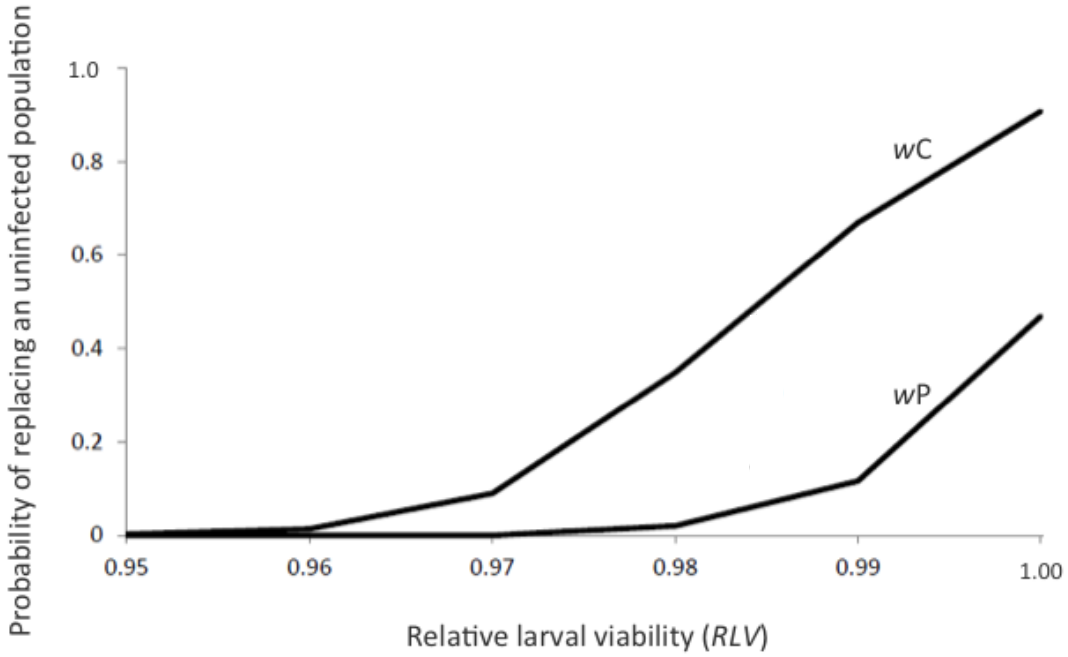


Figure 4.1. The probability of *Wolbachia* invasion into an uninfected population is affected by a fitness cost imposed upon the host by the infection type. Following a single introduction, commensal infections (*w*C) that impose a negligible fitness cost (RLV ≈ 1.0) are likely to invade and replace the uninfected cytotype. With increasing costs to host fitness (i.e., lower RLV values), the probability of *Wolbachia* establishment declines to zero. I simulate host populations in which individuals carry one of two *Wolbachia* types: a commensal (*w*C) and pathogenic (wP) *Wolbachia* type. Delta (Δ) is a fitness multiplier ($0 < \Delta < 1$): a measure of the mosquito fitness associated with a *w*P infection, relative to that of a *w*C infection.

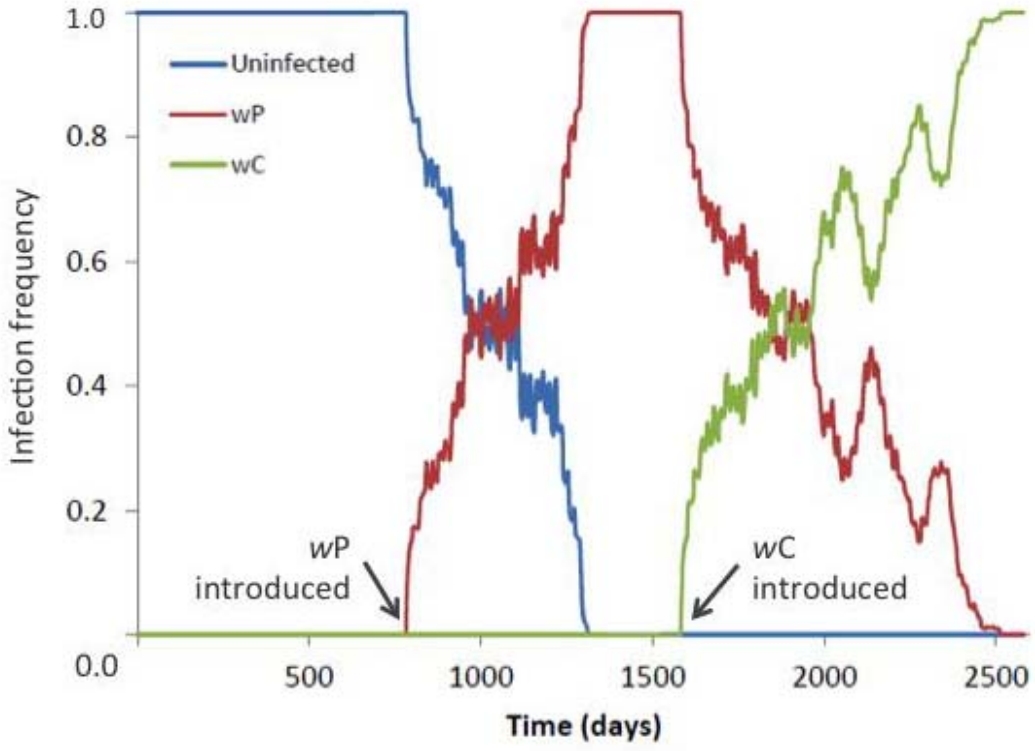
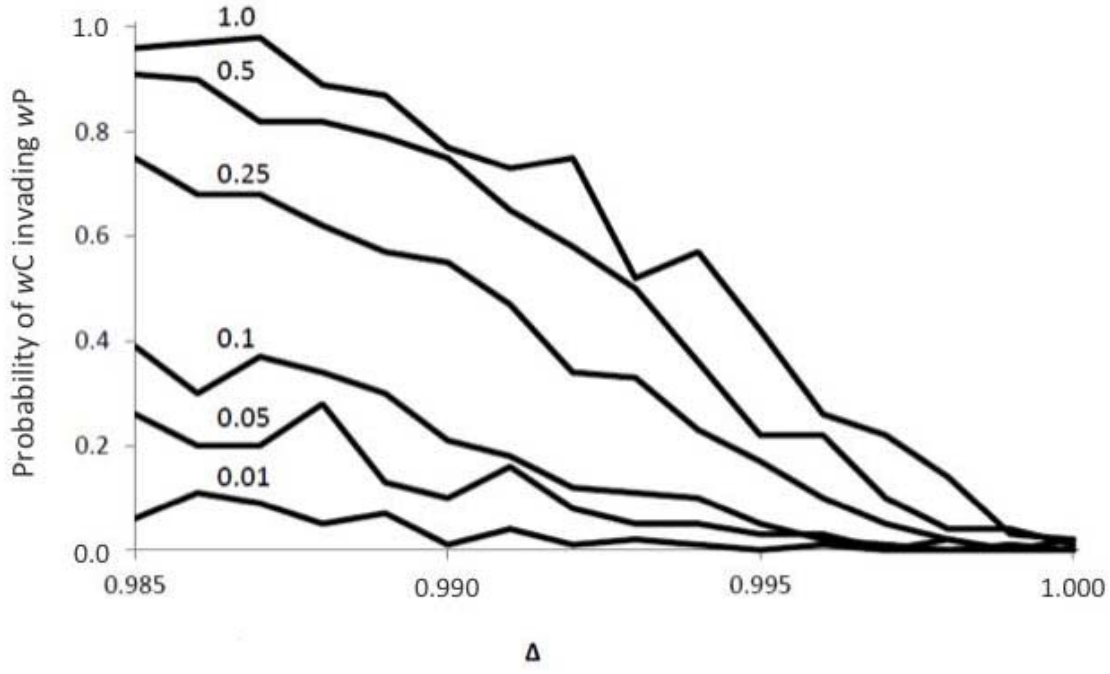


Figure 4.2. An example simulation in which a pathogenic *Wolbachia* infection (*w*P) is introduced into an uninfected population, resulting in a population replacement event, with the *w*P infection increasing to fixation. Subsequently, the introduction of a *w*C infection into the same population results in a second replacement event (Scenario I from the text), with the *w*C infection type replacing the *w*P infection. For the simulation, Δ = 0.991, RLV = 0.999, $IF = \Pi = 0.5$.

a


Probability of wC invading wP
1.0
0.8
0.6
0.4
0.2
0.0
1.0
0.5
0.25
0.1
0.05
0.01
0.985
0.990
0.995
1.000
Δ

b

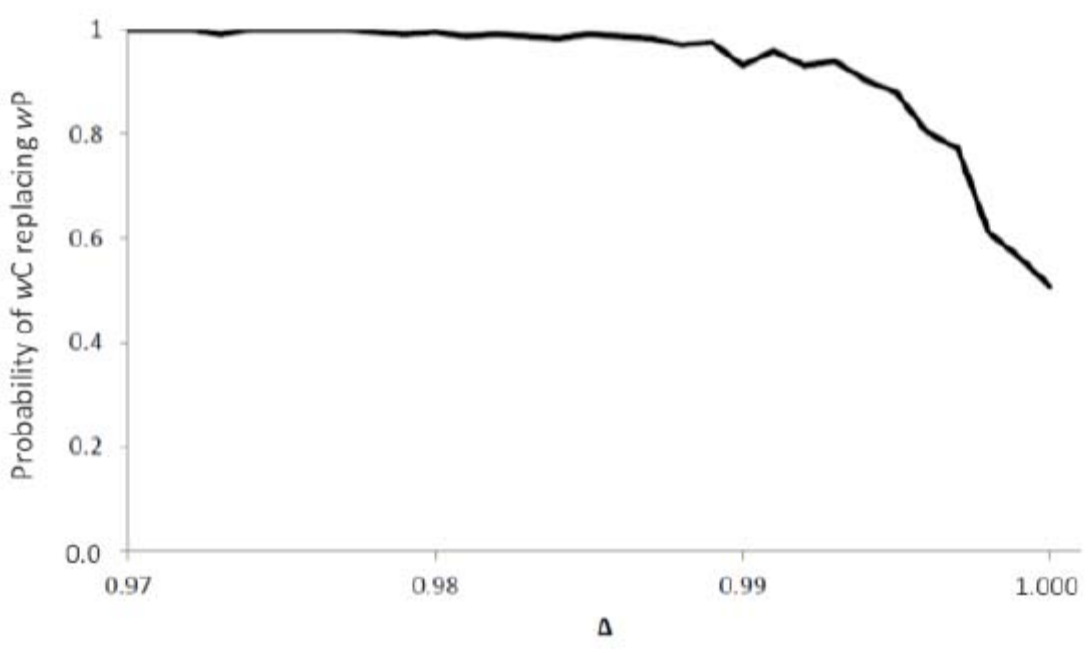

Probability of wC replacing wP
1
0.8
0.6
0.4
0.2
0.0
0.97
0.98
0.99
1.000
Δ

Figure 4.3. Probability of population replacement in different scenarios versus decreases in relative fitness, Δ. In (a) I simulate introductions (Scenario I from the text) of a commensal *Wolbachia* type (*w*C) into a population that is uniformly infected with a pathogenic infection type (*w*P). Delta (Δ) is a fitness multiplier ($0 < \Delta < 1$): a measure of the mosquito fitness associated with a *w*P infection, relative to that of a *w*C infection. At lower Δ values (*i.e.*, the *w*P infection is substantially more costly to its host, relative to the *w*C infection type). In (b) I simulate Scenario II from the text, which is the probability of the *w*C infection replacing a *w*P infection, if equal populations of each are established sympatrically.

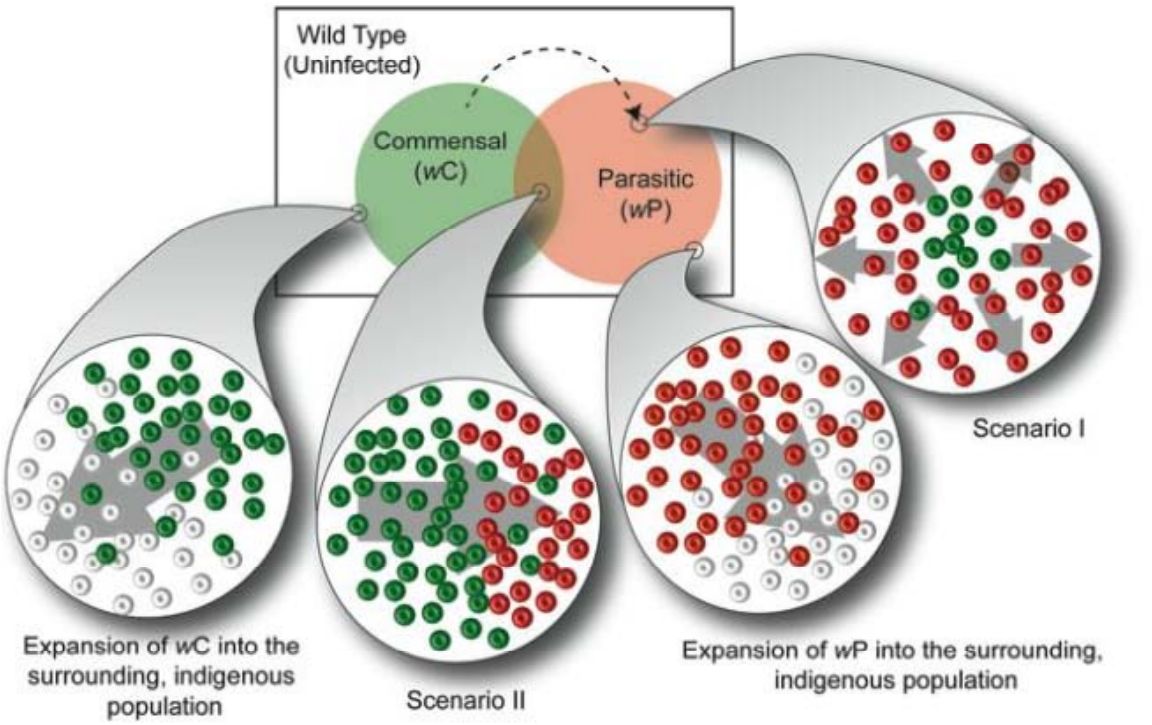


Figure 4.4. Application of the model to existing releases of the *w*Mel and *w*MelPop infections into different cities in Australia. The indigenous, wild type *Ae. aegypti* population is uninfected (white). The *w*Mel infection type has reduced host fitness cost relative to the *w*MelPop infection, analogous to a *w*C (green) and *w*P (red) infection type, respectively. The introduction of *w*Mel infected females into a population infected by *w*MelPop (Scenario I from the text) can result in the spread of *w*Mel and replacement of wMelPop. While released into different locations, one or both of the infections may spread into the surrounding uninfected population, leading to the potential overlap in their distributions (Scenario II from the text) and replacement of the *w*MelPop infection by the *w*Mel infection.

Chapter Five

Analyzing the stability of population replacement by *Wolbachia*-infected individuals

Introduction

Wolbachia pipientis is a maternally-transmitted endosymbiont that is able to manipulate arthropod reproduction to promote infection (Werren et al. 2008). In this way, *Wolbachia* infections can spread rapidly through uninfected populations, a phenomenon known as population replacement (Hoffmann et al. 1990, Turelli and Hoffmann 1991). Specifically, population replacement is the invasion and establishment of a *Wolbachia* infection into an uninfected population (Turelli 2010). Population replacement is facilitated by the reproductive manipulations associated with *Wolbachia* (Werren 1997). There are four reproductive manipulations, but cytoplasmic incompatibility, known as CI, is the most common. CI provides a relative reproductive advantage to *Wolbachia* infected females by sterilizing matings between *Wolbachia* uninfected females and *Wolbachia* infected males (Mercot and Charlat 2004). CI has been called a "lock and key" system where *Wolbachia* infected sperm are "locked" and only eggs with the required "key" can create viable embryos (Poinsot et al. 2003). The CI phenotype is a gene drive mechanism that moves *Wolbachia* into uninfected populations (Marshall 2009).

Recent biological control strategies would use the reproductive manipulations of *Wolbachia* to alter disease vector populations (Walker et al. 2011). Two biological control strategies have been proposed using *Wolbachia*. The first strategy, an extension of

the incompatible insect technique, requires the inundative release of *Wolbachia* infected males into a population (Chambers et al. 2011, Brelsfoard and Dobson 2012). These males are incompatible with an uninfected population, thus producing sterile matings that reduce mosquito populations (O'Connor et al. 2012). A second strategy, currently being implemented in Australia, proposes the release of mosquitoes infected with *Wolbachia* into an uninfected population resulting in population replacement via CI-inducing *Wolbachia* (Hoffmann et al. 2011).

Both biological control programs rely on the transfer of *Wolbachia* infections between insect. *Aedes* mosquitoes, which vector several significant human pathogens, have been infected with novel *Wolbachia* types through two different mechanisms: microinjection (Xi et al. 2005b) and interspecific hybridization (Brelsfoard and Dobson 2007). The transfer of *Wolbachia* infections between hosts is associated with the development of several phenotypes. For example, novel infections induce physiological change within insects, such as increasing reactive oxygen species production or upregulating immune-related genes (Pan et al. 2012, Pinto et al. 2012). Novel *Wolbachia* infections are also linked to decreases in many human pathogens including Dengue virus (Kambris et al. 2010, Walker et al. 2011, Blagrove et al. 2012, Pan et al. 2012, Weiss et al. 2012). Because novel *Wolbachia* infections are associated with human pathogen interference, they are currently being evaluated as a disease control strategy (Hoffmann et al. 2011).

Aedes aegypti are naturally uninfected with *Wolbachia*, making the control of this important vector species possible with both biological control strategies. The current

Dengue Virus control program ongoing in North Queensland, Australia has introduced two new *Aedes aegypti* mosquito strains into the field, each with a novel *Wolbachia* infection (see http://www.eliminatedengue.com; January 16, 2013). The first strain, which has subsequently established in two release sites is infected with a relatively benign *Wolbachia*, and provides intermediate Dengue interference (Hoffmann et al. 2011, Cyranoski 2012). The second infection is more pathogenic and associated with reductions in longevity, but provides complete Dengue interference (Walker et al. 2011, Cyranoski 2012). However, theoretical experiments predicted the release of highly pathogenic infections would not result in population replacement due to the fitness costs associated with infection (Turelli 2010, Barton and Turelli 2011).

The dynamics of a *Wolbachia* invasion are well documented empirically and theoretically (Caspari and Watson 1959, Fine 1978, Turelli and Hoffmann 1991, Rasgon and Scott 2004, Barton and Turelli 2011, Crain et al. 2011). However, the loss of a *Wolbachia* infection once it has established has not been examined thoroughly (Dobson 2003). The stability of *Wolbachia* infections is critical to biological control programs utilizing this bacterium. In this chapter, I consider reverse population replacement, defined as the invasion of uninfected individuals into a population of stably-infected individuals. Pathogenic *Wolbachia* infections are subject to antagonistic selection forces. While high levels of CI promote infection with *Wolbachia*, high fitness costs favor the loss of infection in a population. To examine this theoretically, I vary parameters that could affect population replacement and determine the probability of reverse population replacement for different *Wolbachia* infections. Further theoretical analyses compare four

different population replacement scenarios and examine combinations of parameters that lead to replacement by an invading infection type.

Methods

Reverse population replacement

A previously published model described the development of an explicit population dynamic model, which estimated the invasion of *Wolbachia*-infected individuals into an uninfected population (Crain et al. 2011). This model was modified such that the probability of reverse population replacement could be determined. Specifically, populations were initiated with *Wolbachia* infected embryos rather than uninfected embryos. As defined previously, *Wolbachia* infections were characterized by four parameters: the level of cytoplasmic incompatibility, *CI*; the maternal inheritance rate, *MI*; the relative fecundity of infected females to uninfected females, *RF*; and the relative larval viability of infected larvae, *RLV* (See Table 5.1 for parameter definitions and initial values).

Simulations began with *Wolbachia*-infected embryos, and the population was allowed to self-regulate for 800 days. After 800 days, uninfected females were introduced into the population at a variable rate, relative to the total adult populations. The parameter *REL* was defined as the introduction ratio of uninfected females relative to the total infected adult population. When *REL* = 1, it is equivalent to a 1:1 introduction ratio, uninfected to infected individuals. After the introduction event, the population is

simulated for an additional 1000 days. At the end of all simulations, the infection frequency of the population is determined and recorded.

To determine the probability of reverse population replacement, 1000 simulations of the modified model were run at each 0.01 interval from zero to one for all parameters listed in Table 5.1. One parameter was varied singly while all other parameters were held constant at their initial value. The number of reverse population replacement events was recorded at each interval, where a reverse population replacement event occurred when there were no *Wolbachia* infected individuals in any life stage during a single simulation.

Population replacement contours

Previous models have examined the variance of single parameters and the effect of parameter variation on population replacement (Crain et al. 2011). However, factors influencing *Wolbachia* invasions and population dynamics vary simultaneously. Therefore, a series of simulations was developed to investigate parameter-by-parameter interactions for four replacement scenarios across three different models. The RLV (Chapter 3), *w*P-*w*C (Chapter 4) and reverse population replacement model described here were used to determine the probability of population replacement when *RLV* is varied in tandem with the introduction ratio, Π. (Note that the initial infection frequency, *IF*, defined in the RLV model, is included in the latter defined introduction ratio as is the *REL* parameter.) These simulations vary both parameters uniformly and record the number of population replacement events occurring in 250 replicates.

Results

Reverse population replacement

More than 500,000 simulations were used to estimate the probability of reverse population replacement (*i.e.* the invasion of uninfected individuals into a *Wolbachia*-infected population). Of the five parameters examined here, *CI* and *REL* had negligible effects on the probability of reverse population replacement (Figure 5.1). No reverse population replacement occurred at the highest introduction ratio examined (*REL* = 1.0) when *Wolbachia* infections carried no additional costs and had high levels of CI and MI. Furthermore, uninfected individuals could not invade an infected population even at increased introduction ratios (*REL* = 2.0, a 2:1 uninfected to infected release ratio). When there is no effect of CI (i.e. CI = 0), there are rare reverse population replacement events. Specifically, when the parameter value of *CI* is near zero, it is possible for reverse population replacement to occur (maximum probability of 0.009 at *CI* = 0.01).

The remaining three parameters had strong effects on the probability of reverse population replacement. When *Wolbachia* decreases the relative fecundity of infected females substantially (*RF* < 0.6), the probability of reverse population replacement is high (Figure 5.1). Similarly, if the *Wolbachia* infection has low maternal inheritance (MI < 0.8), the probability of reverse population replacement is also high. However, *Wolbachia* infections that reduce relative larvae viability (*RLV*) are subject to reverse population replacement at higher values relative to other parameters such that if *RLV* is less than 0.92, uninfected individuals have better than a 0.5 probability of reverse population replacement.

Contours of population replacement

Contour plots were generated to illustrate the probability of replacement when parameters Π and *RLV* are varied in tandem for all three models (Figure 5.2). Additional simulations were used to generate a population replacement contour for the invasion of pathogenic *Wolbachia* infections (*w*P) into an uninfected population. To simulate *w*P infections, the original *RLV* model was modified to include the relative cost multiplier Δ (see Chapter 4, Figure 5.2b). Population replacement of an uninfected population by a commensal *Wolbachia* infection (*w*C) occurs most frequently when both *RLV* and Π are high. However, the probability of population replacement decreases sharply with *RLV*. While higher Π increases the probability of population replacement, the effect of increasing Π beyond approximately 0.5 produces diminishing returns, *i.e.* the rate of increase for population replacement as a function of Π slows (Figure 5.2a). Figure 5.2b, illustrates the probability that a *w*P infection would invade an uninfected population. Similar to *w*C infections, replacement will only occur at combinations of high *RLV* and high Π, however the probability of population replacement is always lower for *w*P infections, relative to *w*C, due to the additional cost of infection (Δ).

Figures 5.2c and 5.2d demonstrate the probability that an already established *Wolbachia*-infection is replaced by either uninfected individuals or individuals with a new, compatible *Wolbachia* infection. The loss of infection produces different dynamics relative to the invasion of *Wolbachia*-infected individuals into an uninfected population. Specifically, infection is lost when it becomes costly, resulting in increasing probabilities as parameter values are lowered. Assuming high levels of CI, uninfected individuals will

invade only when *RLV* is approximately 0.9. The introduction ratio, Π, does not affect the probability of population replacement once Π has reached 0.1. Contrastingly, if a compatible *Wolbachia* strain is introduced into an already infected population (*i.e.* there is no CI when individuals of different infection types mate), population replacement occurs at much higher *RLV* values (Figure 5.2d). Without high CI, the probability of population replacement increases with higher Π asymptotically.

In all simulations, Π is less sensitive than RLV. Π demonstrates a Type 2 functional response, such that rate of increase of Π decreases as the probability of replacement approaches 1. RLV exhibits strong threshold behavior (Type 3 functional response).

Discussion

The evolutionary success of *Wolbachia* relies on the stable association between the endosymbiont and its host. Here, I have evaluated reverse population replacement and multiple replacement scenarios to better understand how *Wolbachia* infections invade new host populations and the mechanisms by which they persist. By simulating the release of uninfected individuals into a *Wolbachia*-infected population, I was able to calculate the probability of reverse population replacement, which can be a measure of infection stability. Of the five parameters evaluated, *RLV* was the most sensitive parameter influencing whether reverse population replacement occurred. At parameter values above approximately 0.92, infections are stable despite reduced larval viability.

However, when *RLV* is below 0.92, uninfected populations are able to reinvade. A broad analysis of all three models introduced in this dissertation highlighted interesting trends across population replacement scenarios. Generally, *RLV* is a highly sensitive parameter, and minor variations can affect the probability of replacement. Also, the number of individuals released into a population is only significant when the number of new individuals is low. Beyond a certain introduction rate, the probability of invasion does not change substantially despite increases in the introduction rate.

The relative importance of all parameters in reverse population replacement is similar when compared to traditional population replacement (Crain et al. 2011). CI is shown to have little effect on reverse population replacement (Figure 5.1), when other parameter values are high. As a result, *Wolbachia* infections that are fixed in natural populations are predicted to be stable even when there is no CI. In nature, some *Wolbachia* infections are known to be stable in the absence of CI (Hoffmann et al. 1996). The establishment and stability of such strains is not well understood, but it is hypothesized that these infections may provide additional viral protection to their hosts (Fenton et al. 2011). However, I have shown that non CI-inducing *Wolbachia* can invade uninfected populations largely as a result of genetic drift in small populations (Crain et al. 2011). The study here suggests that a non CI-inducing *Wolbachia* strain, can be stable through high maternal inheritance and no substantial fitness cost.

RLV, RF, and *MI* are the most sensitive parameters dictating reverse population replacement (Figure 5.1). Interestingly, the results here suggest that it is more difficult to invade a population that is infected rather than an uninfected population. Table 5.2 lists

the five key parameters defined here and in Chapter 3, and the corresponding value for each parameter where replacement is predicted in >50% of all simulations. The parameter values are higher in Chapter 3, which suggests that invading infections are successful when they are associated with low costs. However, if CI-inducing *Wolbachia* infections have reached fixation in a population, and the level of CI is high, infections can cause relatively high costs while still being stable. Typically, stable infections in natural populations are commensal or beneficial (Dobson et al. 2002a, Dong et al. 2007). Furthermore, studies demonstrate that *Wolbachia* evolves with its host over time and the level of costs inflicted on the host is reduced (Weeks et al. 2007, McMeniman et al. 2008). Results here do encourage the biological control strategies currently being used in Australia, since those programs are releasing costly infections that also cause Dengue interference (Hoffmann et al. 2011, Walker et al. 2011). If the release of *Wolbachia* infected individuals is coupled with other integrated pest management strategies, then perhaps costly infections could be established. Once established, results from the model designed here suggest that those populations should be stable despite the costs associated with infection.

Figure 5.2. illustrates the effect of introduction rate (Π) and *RLV* on the probability of replacement for four different replacement scenarios. This type of analysis was designed to provide general comparisons of *Wolbachia* invasion and stability. Generally, costly infections are poor invaders, even when Π is increased (Figure 5.2a and b). These results are similar to those reported previously (Barton and Turelli 2011, Crain et al. 2011). However, the analysis here highlights the interaction of *RLV* and Π during reverse population replacement and during competition between multiple, compatible

Wolbachia types. (Figure 5.2d is also the probability of reverse population replacement when there is no effect of CI.) When CI is present, population replacement occurs at relatively low *RLV* and Π has little effect on that probability, which suggests that despite substantial costs *Wolbachia* infected populations will prevent reinvasion of uninfected individuals. If there is no effect of CI (Figure 5.2d) population replacement occurs at higher *RLV* values and Π can strongly affect population replacement, *i.e.* less costly infections will still be lost to reinvasion of uninfected individuals because there is no CI.

The role of CI in *Wolbachia* invasions has been considered theoretically (Turelli and Hoffmann 1991, Jansen et al. 2008, Crain et al. 2011). Models predict that CI can facilitate invasion even when CI is not perfect (Turelli and Hoffmann 1991, Crain et al. 2011). However, other studies predict that CI should be lost once a *Wolbachia* infection has established because there would be no selection to maintain CI in a wholly infected population (Tortosa et al. 2010). Results here suggest that CI is critical for stabilizing new *Wolbachia* infections in nature, particularly if new infections are associated with costs. Increased levels of CI prevent reinvasion of uninfected individuals even when costs of infection are high (Figure 5.2c). High levels of CI serve to isolate newly infected populations, which would allow time for the infection and host to coadapt, reducing costs (Weeks et al. 2007, McMeniman et al. 2008).

Periods of isolation may be important in *Wolbachia* invasions, particularly in a metapopulation framework (Reuter et al. 2008, Hancock and Godfray 2012). Small populations could become infected first, and once the infection has coadapted (i.e. lost all costs of infection), gene flow may facilitate the spread of infection to larger populations.

Previous models have suggested that population structure is a significant factor determining if *Wolbachia* infections can invade uninfected populations (Reuter et al. 2008, Hancock and Godfray 2012). However, genetic drift can facilitate population replacement when populations are small (Crain et al. 2011). Once small populations become infected with coevolved *Wolbachia* infections, they may then be able to invade larger populations linked by gene flow (Barton and Turelli 2011).

Modeling approaches such as the one described here are important for scientists and regulators, particularly for disease control (Vavre and Charlat 2012). A priori planning may help mitigate downstream problems. The model here encourages the use of *Wolbachia* as a biological control agent for mosquito populations. Physiologically, *Wolbachia* causes Dengue interference, but high Dengue interference is associated with high costs (Walker et al. 2011). Initially, this led researchers involved in the Eliminate Dengue Project (http://www.eliminatedengue.com; January 16, 2013) to release a less costly infection that provides intermediate Dengue interference because model predicted that costly infections would not establish in mosquito populations (Barton and Turelli 2011). However, releases of the second, more costly strain began early in 2011 (Cyranoski 2012). By introducing two competing strains, researchers may have increased the difficulty in realizing their objective (see Chapter 4).

The study described here suggested that intense integrated pest management schemes involving the release of *Wolbachia* infected individuals with high costs could establish and will be stable if the level of CI between infected individuals and wild-type populations is high. Future studies should be designed to assess the stability of costly

Wolbachia infections in the lab. Such studies could initiate populations of individuals with costly infections, and track infection status over time with known numbers of uninfected immigrants. However, theoretical work here highlights the need for additional laboratory and semi-field studies to better understand these qualitative predictions.

Table 5.1. The definition of key parameters evaluated by the model and the initial value for all simulations. Unless otherwise stated, all values were held constant and one parameter was varied uniformly in a series of simulations.

symbol	Definition	initial value
CI	proportion of embryos not hatching in incompatible CI crosses	0.999
MI	proportion of offspring receiving infection (maternal inheritance)	0.999
RF	relative fecundity of infected females to uninfected females	0.999
RLV	relative larval viability of infected larvae to uninfected larvae	0.999
REL	initial frequency of gravid uninfected females to the total adult population	0.500

Table 5.2. Comparison of parameter values for the reverse population replacement model and the *RLV* model described in Chapter 3 where the probability of replacement exceeds 0.5. Values listed for Chapter 3 show the numerical value at which infected individuals will invade an uninfected population with a probability of 0.5. Reverse population replacement values lists the numerical value where uninfected individuals will invade an infected population with a probability of 0.5. For all parameters, replacement occurs at lower values when considering reverse population replacement, suggesting *Wolbachia* invasion is more difficult than the persistence of an established infection.

symbol	Invasion value (Ch. 3)	Reverse population replacement value
CI	0.28	n/a
MI	0.92	0.79
RF	0.91	0.60
RLV	0.99	0.90
IF/REL	0.18	n/a

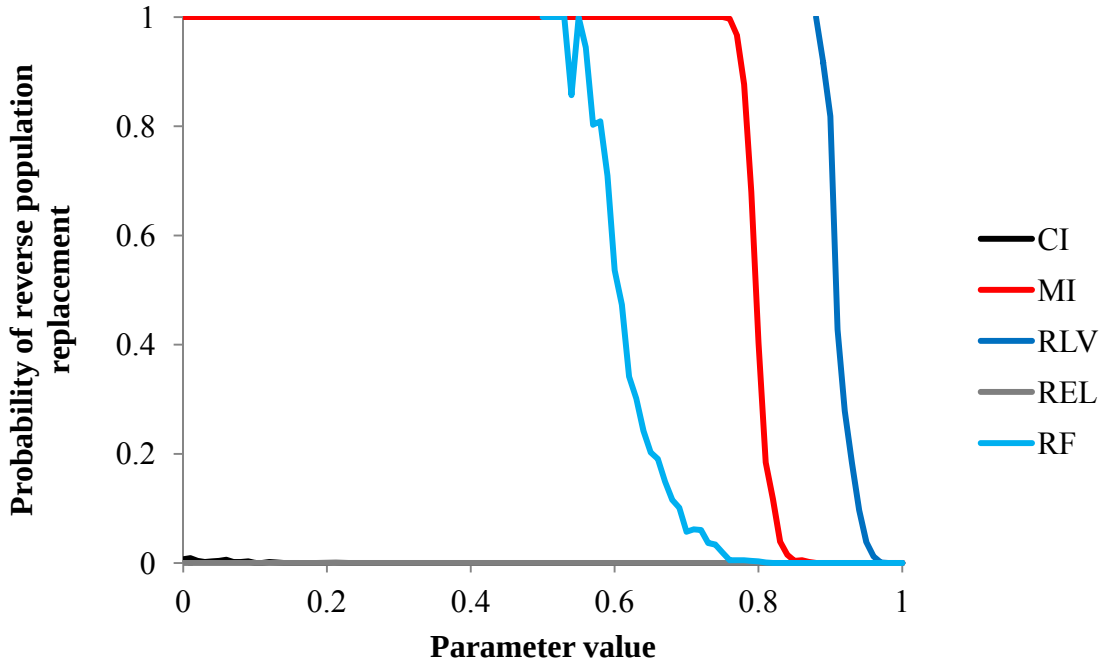


Figure 5.1. The probability of reverse population replacement for five key parameters. Reverse population replacement is defined as the invasion of uninfected individuals into a *Wolbachia* infected population. *CI* is the level of cytoplasmic incompatibility; *MI* is the level of maternal inheritance; *RLV* is the relative larval viability of *Wolbachia* infected larvae; *REL* is the proportional release of uninfected female individuals relative to the total adult population size; *RF* is the relative fecundity of infected females. Each parameter was varied singly from zero to one at 0.01 intervals, holding all other parameters at the value listed in Table 5.1. 1000 simulations were run at each increment.

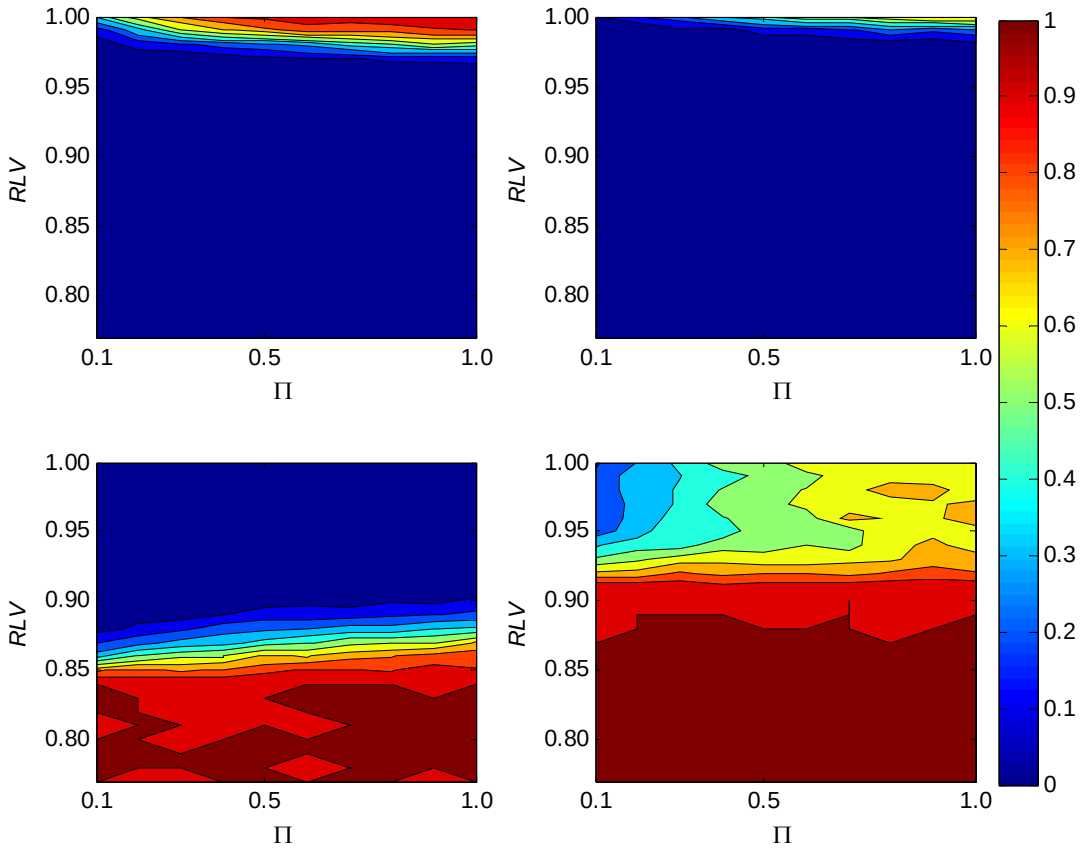


Figure 5.2. Contour plots illustrating the probability of replacement for different scenarios when both relative larval viability (*RLV*) and the introduction rate (Π) are varied in tandem. Red colors represent areas where the combination of the two parameters results in high probabilities of population replacement. Blue colors represent areas where the combinations of the two parameters results in low probabilities of population replacement. The four panels are: a) traditional *Wolbachia* replacement where a *w*C infection invades an uninfected population; b) invasion of a *w*P infection, which is associated with higher costs relative to *w*C infections; c) reverse population replacement of a *w*C infection with high levels of CI; and d) invasion of a *w*C infection into a *w*P-infected population. Generally, *RLV* is important for determining the probability of a

replacement event. Introduction rates, Π, are less important, *i.e.* increasing Π does not increase the probability of replacement in all cases. Also, the importance of CI is demonstrated in c) and d). c) demonstrates reverse population replacement with high CI, but d) demonstrates reverse population replacement when no CI occurs. Costly infections are stable because CI can help eliminate migrant uninfected individuals. When CI is not present, reverse population replacement occurs more frequently.

Chapter Six

The competitiveness of two strains of *Aedes aegypti* with artificial *Wolbachia* infections relative to uninfected mosquitoes

Introduction

Wolbachia pipientis is a well-known bacterial endosymbiont of insects that manipulates its host's reproduction to promote infection. There are four recognized reproductive manipulations associated with *Wolbachia,* but cytoplasmic incompatibility (CI) is observed in most species (Werren et al. 2008). CI occurs when karyogomy failure leads to the arrest of early embryonic development (Charlat et al. 2001). Specifically, CI promotes infection of *Wolbachia* by selectively sterilizing uninfected females in a population who mate with infected males (Duron et al. 2012). This process creates an effective two-to-one reproductive advantage for infected females in the population, which works to drive *Wolbachia* into uninfected host populations (Sinkins and Gould 2006). The invasion and establishment of *Wolbachia* into a previously uninfected population is called population replacement, and has been documented in natural and artificial populations (Turelli and Hoffmann 1991, Xi et al. 2005a, Walker et al. 2011).

Population replacement has been identified as a potential disease control strategy (McMeniman et al. 2009, Hoffmann et al. 2011, Walker et al. 2011). By replacing a population of competent vectors with incompetent vectors, disease control can be obtained while not altering the ecology of a system (Hurst et al. 2012). Traditionally in disease control, population replacement events are facilitated by a gene drive mechanism, but also require the introduction of a transgene into the target vector (Huang et al. 2007).

While some cases have been successful, genetic modification of disease vectors is not well received by the general public and the ability to achieve long term control of disease via a transgene is debatable (Huang et al. 2007, Phuc et al. 2007, Harris et al. 2012, McNaughton 2012). However, *Wolbachia* is an excellent candidate for such a control program because the presence of novel *Wolbachia* infections increases insect immune responses, and limits many human pathogens, without genetic modification (Kambris et al. 2009, Kambris et al. 2010, Pan et al. 2012). The level of disease control varies with infection and with the disease vector considered, but field releases of *Wolbachia*-infected are occurring in Australia to help control Dengue virus (Hughes et al. 2011, Walker et al. 2011).

It is important to understand population replacement thoroughly within disease control programs. Previous theoretical studies have been developed to evaluate the probability of population replacement of different *Wolbachia* infections (Turelli and Hoffmann 1991, Jansen et al. 2008, Crain et al. 2011, Hancock et al. 2011). In these studies, infections are assumed to be costly and the model evaluates conditions under which the infection will spread. For example, infections that carry relatively small fecundity costs are still predicted to invade populations, so long as the initial infection frequency is large enough (Turelli 2010). Further, some models have hypothesized that costs can occur across insect life stages (Rasgon and Scott 2004, Crain et al. 2011, Hancock et al. 2011). Crain et al (2011) defined a parameter called the relative larval viability (*RLV*), which was identified as the most important parameter affecting the probability of population replacement. *RLV* is the relative survival of infected larvae

compared to uninfected larvae. While the results from Crain et al. (2011) are interesting, those results have not been verified empirically.

Few studies have documented the effect of *Wolbachia* on the larval dynamics of insects. Gavotte et al. (2009) demonstrated that male and female mosquitoes have differential survival when exposed to low and high competition, but this effect does not change with *Wolbachia*. However, another study determined that *Wolbachia*-infected individuals have lower survival relative to uninfected individuals when competition is high and there is a mix of infected and uninfected larvae in a common habitat (Gavotte et al. 2010). In Lepidoptera, infection with *Wolbachia* increased susceptibility of larvae to a viral pathogen, leading to decreased survival (Graham et al. 2012). Further studies demonstrate that larval survival due to predation is not affected by *Wolbachia* infection (Hurst et al. 2012). Chapter 2 of this dissertation demonstrates that novel *Wolbachia* infection is associated with decreased larval survival. Despite the growing literature documenting effects of *Wolbachia* on immature insects, few studies have attempted to address how population replacement may be affected by these results.

Here, I describe an experiment to document the change in *Wolbachia* infection frequency over one generation in various population replacement scenarios. Mosquitoes infected with two different *Wolbachia* types were introduced into uninfected populations at known ratios and the change in infection frequency was monitored between generations. Further, a simple mathematical equation was derived to predict the change in the frequency of infection given differences in net reproductive rate between infected and uninfected individuals. The results of this study are discussed in relation to theoretical

predictions of population replacement and how they may affect ongoing disease control programs in Australia.

Methods

Mosquitoes

For this experiment, three strains of *Aedes aegypti* were selected from laboratory colonies. Waco is a wild-type, uninfected strain of *Ae. aegypti* that has been described previously (Xi et al. 2005b, Xi et al. 2005a). WB1 is a strain of *Ae. aegypti* that is infected with the *w*AlbB *Wolbachia* infection, which is naturally occurring in *Aedes albopictus* and is considered a commensal infection with no documented cost of infection (Xi et al. 2005a). PGYP1 are *Ae. aegypti* mosquitoes infected with the *w*MelPop *Wolbachia* infection, which is pathogenic to its host inducing shortened longevity (Min and Benzer 1997, McMeniman et al. 2009). Prior to the initiation of the experiment, the infection status of each strain was determined by PCR and restriction enzyme digest based on previous publications (Zhou et al. 1998).

Bioassay

To initialize the experiment, adult female mosquitoes of each strain were bloodfed on a human subject, mated with a male of a similar *Wolbachia* infection and held for 24 hours in a cage. Mosquitoes were monitored for 24 hours to increase the probability that a gravid female mosquito would survive to lay eggs from and eliminate error due to differential female mortality between strains. After 24 hours, 16 female mosquitoes were introduced into a population cage at known ratios of infected to uninfected individuals.

For each *Wolbachia* infected strain, population cages were initialized at five different infection ratios: 1:0, 3:1, 1:1, 1:3, and 0:1 (infected:uninfected female mosquitoes). A specimen cup lined with germination paper was added to allow for oviposition, and a constant supply of 10% sucrose solution was available. Females were given one week to oviposit all eggs from a single bloodfeeding. After one week, egg papers were removed and divided into two pieces with an equal number of eggs. Those eggs were submerged in distilled, deionized water and given an *ad libitum* supply of liver powder solution. Larvae were monitored daily, and additional food was added as needed. Pupae were removed from rearing pans and transferred into a new population cage with a constant sucrose source until all larvae had either pupated or died. Pupae were allowed to eclose into the population cage, and all adults were aspirated from each cage one week after the last pupae were added. For each cage, 10 females were removed and DNA was extracted to test for the presence of *Wolbachia*. There were a minimum of three replicates for each infection ratio listed above for both PGYP1 and WB1 strains.

PCR analysis of infection

DNA was extracted from adult female mosquitoes by using a previously published method (Gavotte et al. 2010). DNA was then amplified using general *Wolbachia* primers 438F (5'-CATACCTATTCGAAGGGATAG-3') and 438R (5'-AGCTTCGAGTGAAACCAATTC-3'). PCR products were visualized on a 1% agarose gel. Individuals who were *Wolbachia* positive had a band appear at approximately 400bp. If individuals were negative for *Wolbachia*, samples were tested using mosquito CO1 primers CO1 f5 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and CO1 r5 (5'-

TAA ACT TCA GGG TGA CCA AAA AAT CA-3') to confirm DNA extraction was successful (Hebert et al. 2003). The infection frequency per 10 samples was recorded and averaged across at least three replicates.

Statistics

All cages, were aspirated and the total number of females was recorded (Table 6.1). By dividing the total number of adult female offspring by the total number of parental female mosquitoes (n=16), the net reproductive rate for each mosquito strain was calculated. Using this estimate of net reproductive rate, I was able to predict the change in infection frequency from one generation to the next for each infection ratio (Equation 6.1):

$$f_{t+1} = \frac{f_t R_{0i}}{f_t R_{0i} + (1 - f_t) R_{0u}} \qquad (6.1)$$

where f_t is the infection frequency at time t, R_{0i} is the net reproductive rate of infected female mosquitoes, and R_{0u} is the net reproductive rate of uninfected female mosquitoes. Equation 6.1 generates a predicted infection frequency for the next generation assuming independent competition among infected and uninfected larvae, i.e. the presence of one type of larvae does not affect the opposite.

Results

Standing population of adult females

When cages were closed and aspirated, the total adult female population size varied between treatments (Table 6.1). The number of females present in the wild-type

only cage (Waco 1:0) had the largest female population size (285.7 ± 9.1). In treatments where infected and uninfected females were mixed, WB1-Waco 1:1 had the largest female population size (274.5 ± 2.6). All other mixed populations had lower total female population sizes respective to the wild-type population.

Net reproductive rate

Net reproductive rate was calculated by counting the total number of adult females produced by the parental generation of females (n = 16) instead of using a life table estimation. The wild-type *Ae. aegypti* strain (Waco) had the highest net reproductive rate, 17.9 (Table 6.2). Both artificially infected strains, WB1 and PGYP1, had significantly lower net reproductive rates ($F_{2,6} = 29.0$, $p < 0.001$), with an R_0 of 7.9 and 9.5 respectively.

Infection frequency

Based on the initial frequencies used to begin each cage, the change in infection frequency from the parental generation to the F1 generation was predicted (Table 6.3). Predicted infection frequencies were calculated from Equation 1 while observed frequencies were determined by the results of diagnostic Wol438 primer PCR reactions (Figure 6.1). For each of the infection ratios examined, the observed infection frequency was higher than predicted for both WB1 and PGYP1 mosquitoes (Table 6.3). The largest difference between prediction and observed infection frequency was at the 1:1 WB1:Waco infection ratio, where the observed infection frequency was more than 20% higher than predicted.

Discussion

Mosquito strains with novel *Wolbachia* infections experience costs and benefits at different life stages. Fitness estimates predict that the artificially infected mosquito strains have a significantly lower net reproductive rate compared with the wild-type strain. However, the increase in *Wolbachia* infection frequency across one generation is larger than predicted in two mosquito strains with artificial *Wolbachia* infections. The increase in infection frequency provides no evidence that artificially infected mosquito strains are less competitive than wild-type strains when competition is low.

Novel *Wolbachia* infections are often associated with negative host phenotypes (McMeniman et al. 2009, Gavotte et al. 2010). If the costs of infection are too high, models predict *Wolbachia* will not spread through a population (Turelli and Hoffmann 1991, Jansen et al. 2008, Crain et al. 2011). Similar dynamics to those described here have been reported previously (Gavotte et al. 2010), and could be important for evaluating population replacement strategies that release *Wolbachia* infected insects. Studies have determined that the competitiveness of *Wolbachia* infected larvae decreases as competition levels increase (Gavotte et al. 2010). Additional theoretical analyses should quantify the relative larval viability of *Wolbachia* infected strains and determine the mechanism that increases infection frequency during low competition scenarios. Larval competition in mosquitoes has been described analytically, and is known to be nonlinear (Dye 1984, Gavotte et al. 2009, Gavotte et al. 2010). Simple theoretical models could be generated to qualitatively assess differences in relative larval viability (via competition for limited resources) and the degree of nonlinearity.

Net reproductive rate is a measure of fitness collected across discrete generations and is commonly associated with life table analyses (Suman et al. 2011, Helinski and Harrington 2012). Instead of estimating net reproductive rate from a life table, full counts of all eclosed adult females were recorded. Wild-type mosquitoes had significantly higher fitness compared to both strains with artificial *Wolbachia* types which had equivalent net reproductive rates (Table 6.2). The result was unexpected because PGYP1 is considered a pathogenic infection and WB1 has been characterized as a commensal infection that causes little to no cost to its host (Xi et al. 2005a). Previous studies found no significant difference in fecundity between WB1 and Waco mosquitoes (Xi et al. 2005a), but data presented here suggest that net reproductive rate differs between WB1 and Waco, suggesting the life histories of the two strains differ. Mosquito populations are highly influenced by intraspecific competition at the larval stage, and the larval dynamics of *Wolbachia* infected and uninfected individuals are known to vary (Gavotte et al. 2010, Hardstone and Andreadis 2012, Walsh et al. 2012). Both artificially infected strains, WB1 and PGYP1, suffer significant fitness costs, which may limit their efficacy as disease control candidates. However, future studies should create detailed life table analyses of theses strains to determine what factors contribute to and exacerbate this reduced fitness.

Horizontal transfer of *Wolbachia* is rarely documented in empirical studies (Heath et al. 1999, De Barro et al. 2011). However, recent analyses predict that the horizontal transfer of *Wolbachia* may occur more frequently than previously predicted (Kremer and Huigens 2011, Watanabe et al. 2012). Phylogenetic analyses determine that *Wolbachia* infections cluster based on shared food sources with phytophogous insects having the

most similar infections (Charlat et al., unpublished). If horizontal transfer of *Wolbachia* is possible, it should promote infection (Kremer and Huigens 2011). In this study, infection frequencies were higher than expected, but it is unlikely that horizontal transfer occurred. Recent experiments determined that the *Wolbachia* strain *w*MelPop is not horizontally transferred from mosquito larvae to several natural predators (Hurst et al. 2012). Because *Wolbachia* is not transferred through this direct association (i.e. predation), it is unlikely that the infection would spread indirectly (i.e. cohabitation). However, future studies could rear infected and marked uninfected individuals in a common environment. Then uninfected individuals could be tested for the presence of *Wolbachia* after some allotment of time.

By developing a theoretical framework whereby empirical studies can be designed, researchers can generate *a priori* hypotheses about their current system (Alphey et al. 2011). Therefore, theoretical models should be fluid and should be redesigned to reflect empirical data when available. The *RLV* Model (Chapter Three) was modified such that uninfected larvae were subjected to decreased larval viability rather than *Wolbachia* infected larvae. Specifically, the linear multiplier *RLV* was applied to uninfected larvae (instead of infected larvae as in Chapter Three), and a series of simulations was conducted. In the first set of simulations, all parameters were held constant at the initial conditions defined in Chapter 3, but *RLV* was set to 1.00 for control simulations. When *RLV* of uninfected larvae is set to 1.00 the probability of population replacement is 0.945 (the approximate maximum described in Chapter Three). When *RLV* of uninfected larvae is lowered to 0.99, population replacement occurs at a probability of 0.975. If *RLV* is further decreased to 0.975, population replacement occurs

at a probability of 0.987. These simulations suggest that the probability of population replacement is increased if infected larvae are more competitive than uninfected. However, the study discussed here also suggests a difference in reproductive rate between infected and uninfected females. There is no method in the *RLV* Model to reduce net reproductive rate directly, but if relative fecundity (*RF*) is reduced proportionally to the difference in reproductive rate, then *Wolbachia* is not predicted to invade despite increases in *RLV* (no population replacement events occurred in 1000 simulations). Additional experiments are needed to determine the factors that influence larval survival in both infected and uninfected individuals. These experiments should examine high, moderate, and low levels of competition in immature mosquitoes and aim to quantify differences in survival.

Wolbachia can be a key component in an integrated pest management regime designed to reduce disease vector populations (Hoffmann et al. 2011). However, *Wolbachia*-host interactions can be taxon specific and may vary temporally (Weeks et al. 2007). Before *Wolbachia* is used in large scale vector control programs, the risks and benefits of such an insect control strategy must be considered (Yeap et al. 2011). Implementation of these strategies requires many studies that range from molecular analyses to field trials and efficacy assays. To understand the totality of such an approach, a theoretical framework is beneficial. As demonstrated here, fluid theoretical frameworks can drive empirical research, but can also reflect recent data. Data here suggest that under certain conditions, infected larvae may be more competitive than uninfected larvae. Before these results are extrapolated to field releases, there needs to be more studies that determine the relationship between density and *RLV*. If *Wolbachia*

infected larvae are more competitive than uninfected larvae in enough scenarios, then *Wolbachia* can be used to manipulate insect vector populations.

Table 6.1. The mean number of eclosing females in each experimental cage. Data shown are the average of at least two replicates (±SE). Waco are wild-type *Aedes aegypti* and are not infected with *Wolbachia.* WB1 are *Ae. aegypti* with a microinjected *Wolbachia* infection (*w*AlbB from *Aedes albopictus*). PGYP1 are *Ae. aegypti* with a microinjected *Wolbachia* infection injected from *Drosophila* (*w*MelPop from *Drosophila melanogaster*).

cage	number of adult females
Waco 1:0	285.7 ± 9.1
WB1 1:0	152.0 ± 24.7
PGYP1 1:0	127.0 ± 7.8
WB1-Waco 3:1	204.7 ± 10.1
WB1-Waco 1:1	274.5 ± 4.5
WB1-Waco 1:3	191.7 ± 15.4
PGYP1-Waco 3:1	113.0 ± 11.0
PGYP1-Waco 1:1	197.5 ± 8.5
PGYP1-Waco 1:3	173.5 ± 5.5

Table 6.2. Net reproductive rate differs between three *Aedes aegypti* strains. Data shown are means ± s.d. (n = 3 replicates). Waco are wild-type *Aedes aegypti* and are not infected with *Wolbachia.* WB1 are *Ae. aegypti* with a microinjected *Wolbachia* infection (*w*AlbB from *Aedes albopictus*). PGYP1 are *Ae. aegypti* with a microinjected *Wolbachia* infection injected from *Drosophila* (*w*MelPop from *Drosophila melanogaster*). Letters represent values that are statistically different.

strain	**net reproductive rate (R_0)**
Waco	17.9 ± 1.0a
WB1	7.9 ± 0.8b
PGYP1	9.5 ± 2.7b

Table 6.3. Predicted and observed infection frequencies for different initial infection ratios (infected:uninfected). Predicted values were calculated using Equation 1and the net reproductive rates in Table 6.1. Observed values were averaged over three replicates (five for WB1 1:1) and were determined by the number of positive samples per ten females at each ratio. *RLV* was determined by dividing the observed value by the predicted value.

	WB1			**PGYP1**	
Ratio	**Predicted**	**Observed**	**Ratio**	**Predicted**	**Observed**
1:0	100	100	**1:0**	100	100
3:1	57.2	93.3	**3:1**	61.5	66.7
1:1	30.8	52.0	**1:1**	34.7	46.7
1:3	12.9	13.3	**1:3**	15.1	20.0
0:1	0	0	**0:1**	0	0

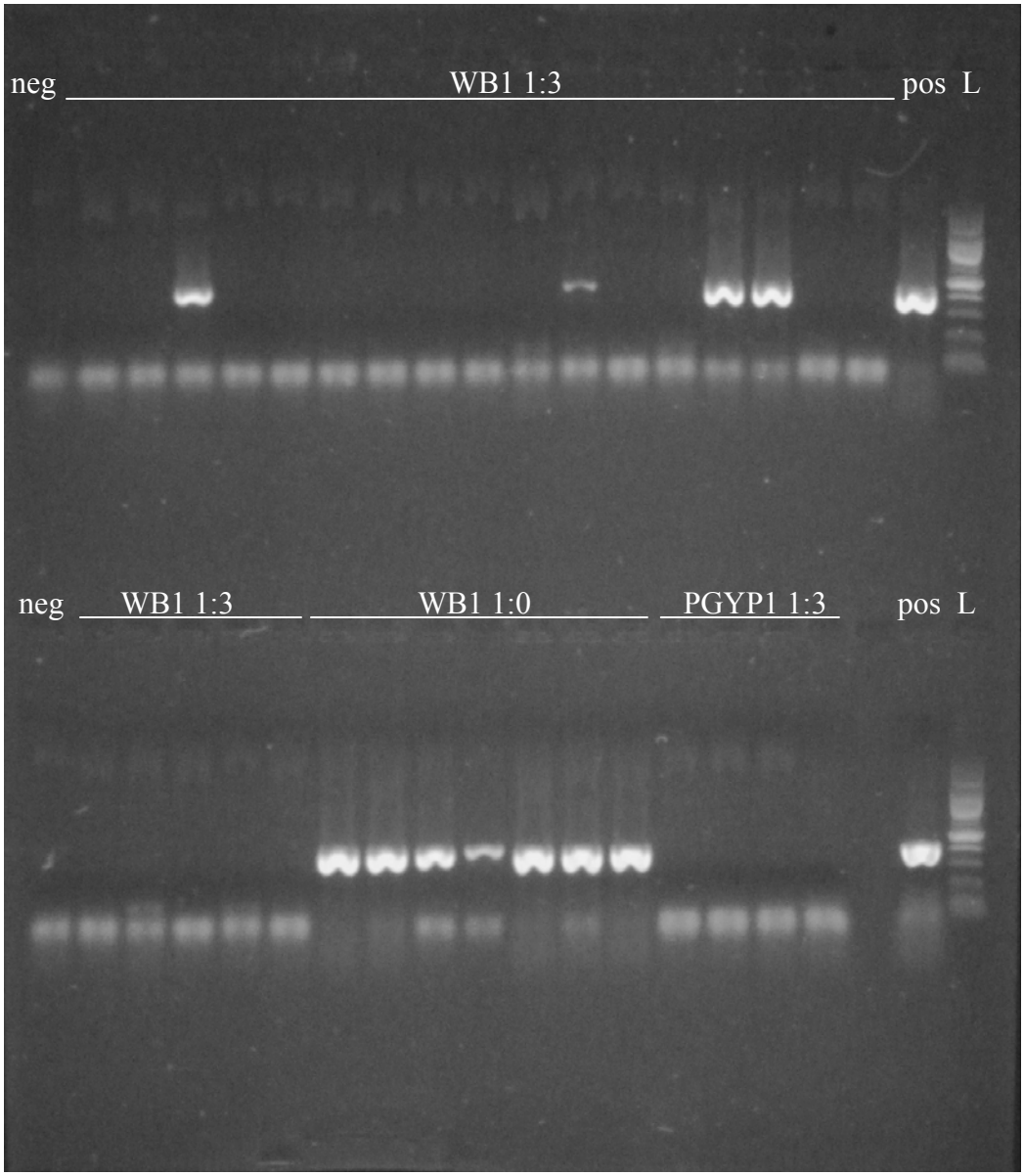


Figure 6.1. PCR products from Wol438 primer set visualized on a 1% agarose gel. Wol438 general *Wolbachia* primers produce a PCR product of 400bp. The gel shown is a compilation of samples across multiple replicates for the WB1 1:3, WB1 1:0, and PGYP1 1:3 treatments. neg is the negative control, pos is the positive control, and L is the 100bp ladder. Each lane is an individual sample consisting of one adult female mosquito.

Chapter Seven

Larval survival of a disease vector decreases after long-term egg storage in a disease vector

Introduction

The number of reported cases of Dengue virus has increased exponentially in recent decades (Guzman et al. 2010). Currently, approximately 2.5 billion people are at risk of contracting Dengue and between 50-100 million cases are diagnosed each year (Guzman and Kouri 2002). There are currently no effective vaccines to prevent Dengue, so controlling its vector, *Aedes aegypti*, is the only viable method to limit new infections (Schmitz et al. 2011). *Aedes aegypti* is a vector of several significant human pathogens, including Dengue virus and Yellow Fever virus (Mackenzie et al. 2004, Tomori 2004). These mosquitoes inhabit both rural and urban environments, and utilize natural and artificial containers (Lounibos 2002).

Recently, disease control programs have been developed based on infections of *Wolbachia pipientis* (Hoffmann et al. 2011, Walker et al. 2011). *Wolbachia* is an obligate, intracellular bacterium that manipulates the reproduction of its host (Werren et al. 2008). There are various reproductive manipulations associated with *Wolbachia* infection, but the most common phenotype is cytoplasmic incompatibility or CI (Charlat et al. 2001). CI causes conditional sterility when uninfected female insects mate with *Wolbachia*-infected males. CI-inducing *Wolbachia* behaves like a gene drive system, and the resulting disease control program is known as population replacement (Huang et al. 2007, Marshall 2009). Critical to the success of population replacement strategies is

pathogen interference, which is a decrease in the prevalence of human pathogens in mosquitoes with artificial *Wolbachia* infections (Moreira et al. 2009, Bian et al. 2010, Kambris et al. 2010, Hughes et al. 2011, Walker et al. 2011, Andrews et al. 2012, Blagrove et al. 2012, Pan et al. 2012). Ultimately, population replacement results in the invasion of *Wolbachia*-infected individuals into uninfected wild-type populations. Due to the pathogen interference, this produces a mosquito population that cannot transmit disease.

Wolbachia infections that cause pathogen interference are also associated with costs to their hosts. In Chapter 2, I demonstrate that larval viability is decreased in mosquitoes that carry artificial *Wolbachia* infections. Further, studies have shown that some *Wolbachia* infections decrease adult longevity (McMeniman et al. 2009). These costs are predicted to decrease the probability that *Wolbachia* establishes in a population (Barton and Turelli 2011, Crain et al. 2011). Because *Wolbachia* invasion is the goal of some disease control programs, factors that may influence invasion need to be evaluated.

In the study presented here, eggs are stored for varying periods of time, and then hatched. Then a consistent number of larvae are tracked and survival to pupation is recorded. Previous studies have demonstrated that there is a reduction in egg hatch associated with increasing periods of dormancy in insect eggs with artificial *Wolbachia* infections (McMeniman and O'Neill 2010). Here that study is extended and I predict that increased dormancy will negatively affect larval survival.

Methods

Mosquitoes

Seven mosquito strains, differing in *Wolbachia* infection status and species, were used for this experiment. Three mosquito strains belonged to the species *Aedes polynesienesis.* The first strain, APM, was collected from natural populations in French Polynesia*,* and has been reared in the laboratory for many generations (Dean and Dobson 2004). A second strain, APMT, was created by tetracycline treating the APM strain, thus clearing the *Wolbachia* infection (Dean and Dobson 2004). The third *Ae. polynesiensis* strain, MTB, was created by the microinjection of *Aedes albopictus Wolbachia* into APMT eggs (Andrews et al. 2012). MTB is stably infected with only the *w*AlbB *Wolbachia* infection from *Ae. albopictus*.

Two mosquito strains of *Aedes albopictus* were examined here. The first strain, WC, is laboratory colony that was recently collected from local field populations in Lexington, KY (38.025841,-84.516584). Similar to the strategy described above, WC was also treated with tetracycline to create an aposymbiotic line, WCT (Dean and Dobson 2004).

Two strains were selected for *Aedes aegypti*. *Aedes aegypti* is naturally free of *Wolbachia* infection, and a wild type strain, Waco, was chosen. Waco was collected from field populations, but has been maintained in a laboratory colony for many generations (Xi et al. 2005a). The second strain was chosen because it is the focus of a disease control program (Walker et al. 2011). The PGYP1 strain is *Aedes aegypti* mosquitoes that are infected with the *w*MelPop *Wolbachia* infection, which was introduced by microinjection (McMeniman et al. 2009).

Bioassay

A population cage of each strain was maintained as previously described (Dean and Dobson 2004). A single egg paper was collected from each cage and stored in standard insectary conditions (~27°C, ~80% relative humidity, 16:8 light:dark cycle). Each egg paper was then divided into three parts. One quarter of each egg paper was hatched in a dilute liver powder solution (0.6g/L, MP Biomedicals LLC, Solon, OH) after one month of storage. The remaining sections of all egg papers were returned to insectary for storage. Hatching egg papers were left for two hours, then 100 first instar larvae were moved using a Pasteur Pippette (BD Biosciences, Franklin Lakes, NJ) to larval rearing pans (dimensions 21 x 21 x 7.5-cm, Pactive, Lake Forest, IL) with 500mL of distilled, deionized water and 200mg of dried liver powder. Two days after each pan was initiated, another 200mg of dried liver powder was added. Larvae were allowed to pupate, and the survival rate of larvae was calculated and recorded. This procedure was repeated one month later by removing another one quarter of all stored egg papers, and hatching them as described above (*i.e.* one quarter of the egg paper was hatched after two months of storage in an insectary). Finally, the remainder of the egg paper was hatched after three months in storage for all strains, and pupal survival was calculated as described above.

Statistics

The experimental design initially considered only *Ae. polynesiensis* strains. Additional strains were added after preliminary experiments to determine if patterns found here were host-specific or occur more broadly. All statistical analyses were performed with JMP 9.0.0 (Cary, NC, USA).

An analysis of variance (ANOVA) was used to determine if there were differences in larval survival between *Ae. polynesiensis* strains over egg age (storage duration). Survival was expressed as a proportion and was arcsine-square root transformed for analysis. There were three factors analyzed here: STRAIN, AGE (which is the age of the egg paper or storage duration), and REPLICATE. Post-hoc means separation was determined by a Tukey HSD test. Additional strains from other species were analyzed similarly.

A final analysis compared larval survival of larvae hatched after three months of storage across all eight mosquito strains. This statistical design was used to determine if the effects of tetracycline treatment and microinjection were broadly similar or if effects differed on a case-by-case basis. An ANOVA was used to determine overall differences in survival, and linear contrasts were designed to determine if there are differences between natural and artificial *Wolbachia* infection states.

Results

Initial analyses determined that there were no significant differences in larval survival due to replication ($F_{2,74} = 0.67$, $p = 0.52$). Therefore, REPLICATE was removed as a factor from further analysis.

There were significant differences in larval survival for the three strains of *Aedes polynesiensis* examined ($F_{8,27} = 9.48$, $p < 0.0001$). STRAIN was a significant factor ($F_{2,27} = 18.63$, $p < 0.0001$), and post-hoc means separation determined that larval survival is higher for wild type (APM) and aposymbiotic (AMPT) mosquito strains than for the microinjected (MTB) strain. Similarly, AGE was also a significant factor in the model

($F_{2,27} = 7.81$, $p < 0.005$). Larvae survived statistically better when they were hatched from egg papers that were stored for one or two months compared to larvae hatched from egg papers that were stored for three months. The interaction of STRAIN and AGE was also significant ($F_{4,27} = 2.84$, $p < 0.05$). Post-hoc means separation determined that the microinjected larvae (MTB) hatched from egg papers stored for three months were significantly lower than all other strain and age combinations (Figure 7.1).

The bioassay and analyses above was extended to strains from *Ae. albopictus* and *Ae*. *aegypti*. For *Ae*. *albopictus*, there was no significant difference in larval survival between tetracycline treated mosquitoes (WCT) and wild-type mosquitoes (WC; $F_{5,11} = 2.90$, $p = 0.07$). A similar statistical model was used to analyze larval survival between two *Ae. aegypti* mosquito strains. There is an overall difference in larval survival ($F_{3,11} = 29.25$, $p < 0.0001$). STRAIN is statistically significant ($F_{1,11} = 53.39$, $p < 0.0001$), where wild-type mosquitoes (Waco) have higher survival than microinjected mosquitoes (PGYP1). AGE was also significant ($F_{1,11} = 21.77$, $p < 0.005$), and larvae that were hatched from two month old egg papers had lower survival relative to larvae hatched from one month old egg papers. Note that three month egg papers were not considered in this analysis because PGYP1 egg papers held longer than two months in storage produced no viable larvae. The interaction of STRAIN x AGE was significant ($F_{1,11} = 12.58$, $p < 0.01$). For post-hoc means separation determined that PGYP1 larvae hatched from two month old egg papers had reduced survival relative to all other combinations (Figure 7.3).

An additional analysis was conducted where I only considered the survival of larvae hatched from egg papers stored for three months (two month for PGYP1 egg

papers since there were no viable larvae beyond that time point) and mosquito strain. This analysis combined mosquitoes across the three different species examined here. There was an overall difference determined by an ANOVA with STRAIN as the lone factor predicting larval survival ($F_{7,19} = 20.35$, $p < 0.0001$, Figure 7.4). Then, post-hoc linear contrasts were used to determine if: 1) tetracycline treatment affected larval survival, 2) microinjection affected larval survival, and 3) if artificial *Wolbachia* infection statuses has lower larval survival compared to the natural infection state. Note that the natural infection state for *Ae. aegypti* is uninfected, meaning no representative of *Ae. aegypti* was analyzed to determine the effect of tetracycline treatment. There was no difference identified between tetracycline-treated strains and their comparative wild-type strain ($F_{1,19} = 0.21$, $p = 0.65$). However, microinjected mosquito strains had lower larval survival when compared to wild-type strains ($F_{1,19} = 57.61$, $p < 0.0001$). Finally, strains that had an artificial *Wolbachia* infection status had significantly lower larval survival when compared to wild-type mosquito strains ($F_{1,19} = 25.96$, $p < 0.0001$).

Discussion

Artificial infection with *Wolbachia* can be associated with costs to the host insect. In *Drosophila* and *Aedes* mosquitoes, a mutated *Wolbachia* infection causes reduced longevity (Min and Benzer 1997, McMeniman et al. 2009). Studies also report that some strains with novel *Wolbachia* infections experience decreased egg hatch when eggs are stored for relatively long periods of time (McMeniman and O'Neill 2010). Here, I examined the survival of larvae that resulted from hatched eggs stored for different durations of time. Larval survival is reduced when eggs from mosquitoes with artificial

Wolbachia infections are stored for relatively long periods of time. There was no detectable difference between wild-type larval survival and aposymbiotic mosquito larvae. Further, *Aedes albopictus* larvae with an artificial *Wolbachia* infection introduced via microinjection did not experience reduced larval survival. For *Aedes polynesiensis* and *Aedes aegypti*, larvae with artificial *Wolbachia* infections introduced by microinjection did exhibit reduced survival relative to the wild-type.

Disease control programs that utilize *Wolbachia* depend on the ability of infection to increase insect immune responses (Brennan et al. 2008, Andrews et al. 2012, Pan et al. 2012). The increase in insect immune response limits human pathogens, creating inefficient disease vectors (Kambris et al. 2010, Hughes et al. 2011, Andrews et al. 2012, Pan et al. 2012). However, the increase in insect immune response is also linked to decreased fitness and negative host phenotypes (Jacot et al. 2004, Jacot et al. 2005). In mosquitoes, negative host phenotypes include reduced longevity in adult mosquitoes and decreased ability to acquire bloodmeals (Suh et al. 2009, Moreira et al. 2011). Furthermore, long periods of dormancy can decrease the egg hatch success of eggs laid by female mosquitoes with artificial infections (McMeniman and O'Neill 2010). The study presented here determined that of the hatching eggs, fewer larvae reach pupation if they harbor artificial *Wolbachia* infections relative to wild-type females. Such a complication could inhibit disease control programs based on population replacement. Theoretical studies have determined that population replacement is highly unlikely when larval survival is reduced (Crain et al. 2011). Although data shown here do not show a direct reduction in larval viability in all cases, it is nonetheless a factor that should be included in future analysis. Particularly, factors like the one described here need to be

addressed in disease control program planning and may help refine estimates of field release rates.

It is not known what process leads to the reduction in larval survival or egg hatch in dormant eggs. The most dramatic negative phenotypes are associated with the *Wolbachia* infection *w*MelPop, which is known to be pathogenic to its host (Min and Benzer 1997). *w*MelPop proliferates outside the ovaries and causes tissue degeneration. However, most negative phenotypes occur in older adults when enough time has elapsed that *Wolbachia* has sufficiently over replicated (McMeniman et al. 2009, Suh et al. 2009, Moreira et al. 2011), suggesting negative phenotypes seen before later adulthood are not attributable to over replication of *Wolbachia*. Other studies have demonstrated that immune upregulation is associated with fitness tradeoffs in some insects (Jacot et al. 2004, Jacot et al. 2005). The mechanism by which larval viability is decreased was not examined here, but future studies should elucidate the physiological mechanism of the reduction in viability. Searching for gene upregulation between strains, then linking those data to fitness assays, may help determine if there are fitness/immunity tradeoffs.

The study described here was designed to determine if there are costs to larval viability when eggs are stored for long periods. Results varied between strains and across species. The results suggest that *Wolbachia* host interactions are case specific. Future studies should look to move the same *Wolbachia* infection into different species to determine if the same *Wolbachia* infection behaves similarly despite the host background. *Wolbachia* host interactions are also time-sensitive. Specifically, the longer that *Wolbachia* and its host are in a symbiosis, the less pathogenic the infection becomes.

Long term serial passage in a cell cultures have expedited this process. Further studies should also examine if larval survival is decreased in “co-evolved” relationships.

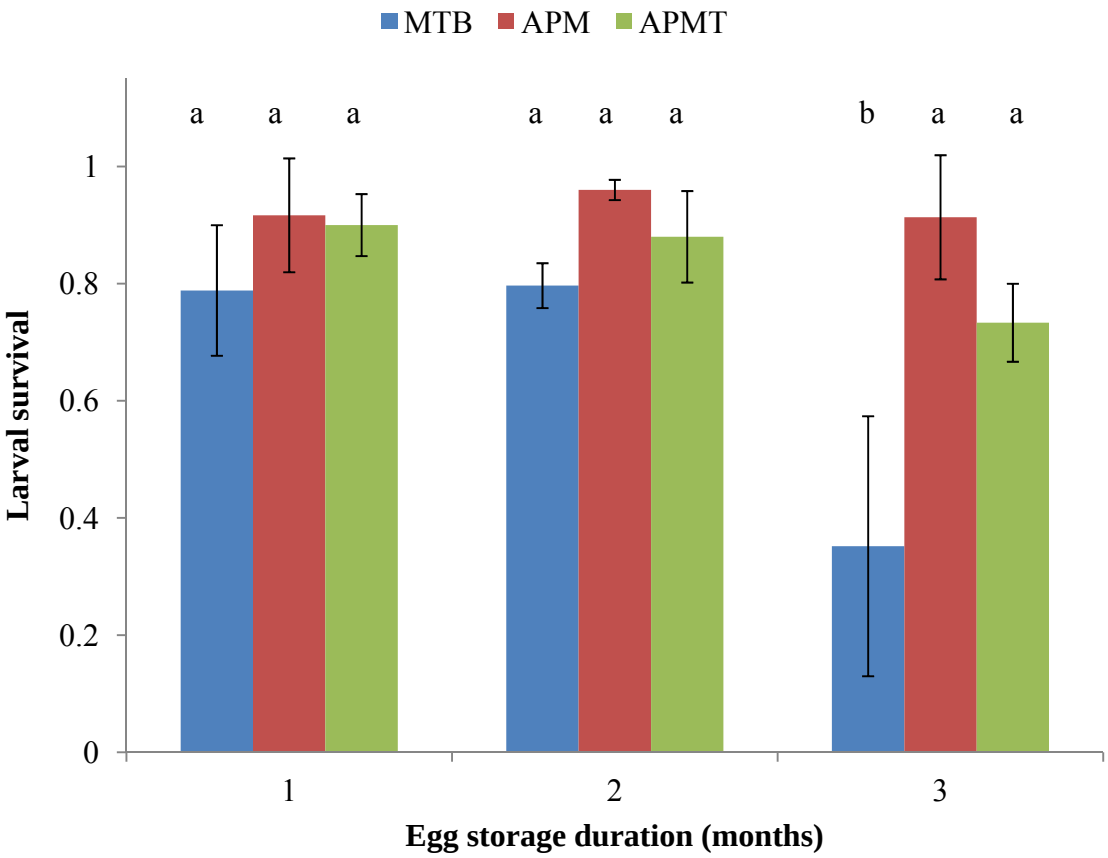


Figure 7.1. Differences in larval survival for three *Aedes polynesiensis* strains hatched from egg papers stored for one to three months. There are no significant differences in larval survival between APM (wild-type), APMT (aposymbiotic), and MTB (microinjected) mosquitoes during the first or second month of egg paper storage. After three months of storage, larvae hatched from MTB egg papers experience reduced survival relative to APM and APMT larvae. Bars with the same letter are not statistically different (Tukey HSD; $p < 0.05$). Graph shows the average proportion of larvae surviving ± standard deviation (n = 3 replicates).

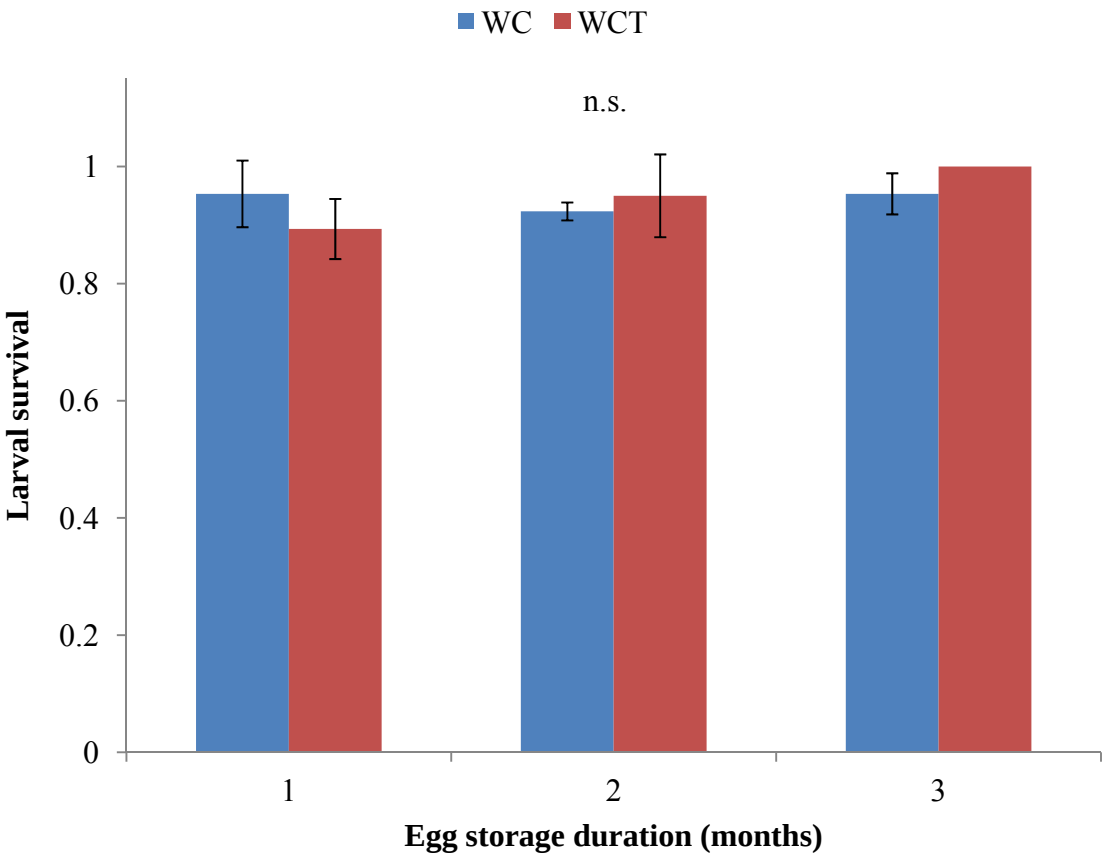


Figure 7.2. Differences in larval survival for two *Aedes albopictus* strains hatched from egg papers stored for one to three months. There are no significant differences in larval survival between WC (wild-type), and WCT (aposymbiotic) mosquitoes after one to three months of storage. Graph shows the average proportion of larvae surviving ± standard deviation (n = 3 replicates).

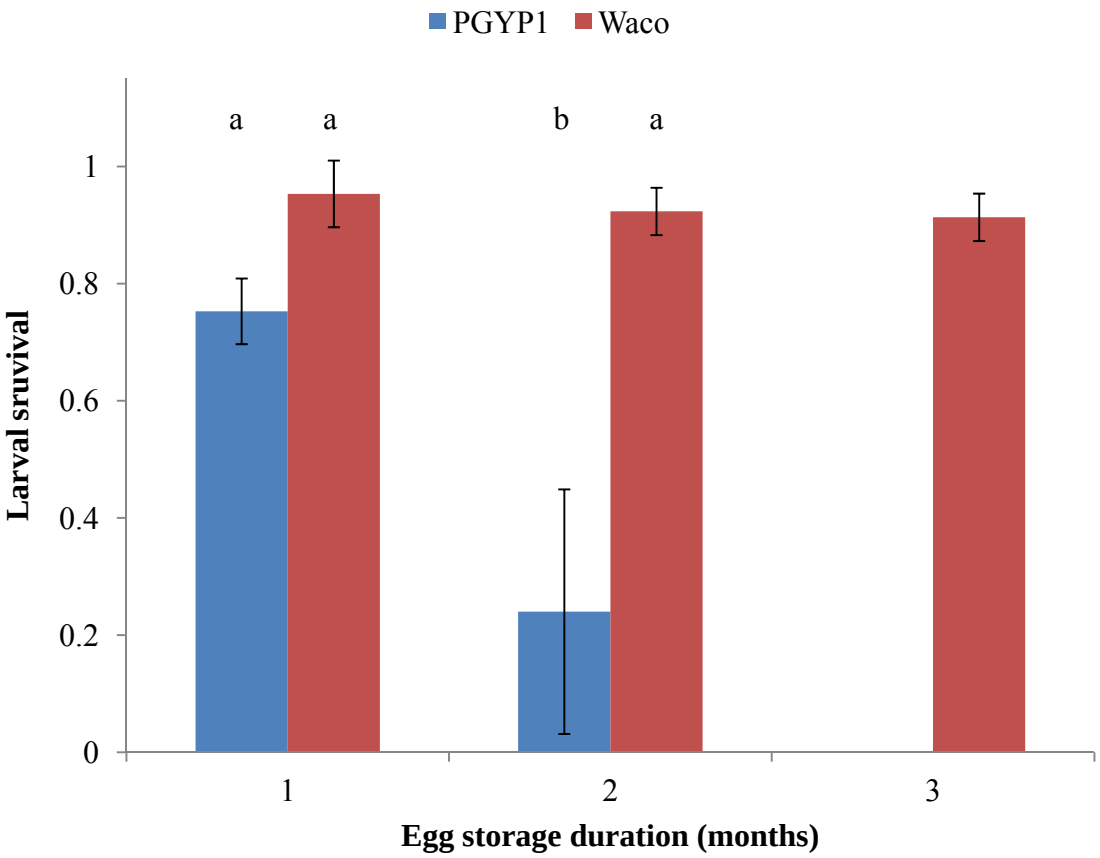


Figure 7.3. Differences in larval survival for two *Aedes aegypti* strains hatched from egg papers stored for one to three months. There are no significant differences in larval survival between Waco (wild-type) and PGYP1 (microinjected) mosquitoes after one of storage. However, Waco larvae that hatch from an egg paper stored for two months survive significantly better than PGYP1 larvae. Bars with the same letter are not statistically different (Tukey HSD; $p < 0.05$). Graph shows the average proportion of larvae surviving ± standard deviation (n = 3 replicates). (Note that no PGYP1 larvae hatched from egg papers stored for three months, therefore data from month three were not analyzed statistically).

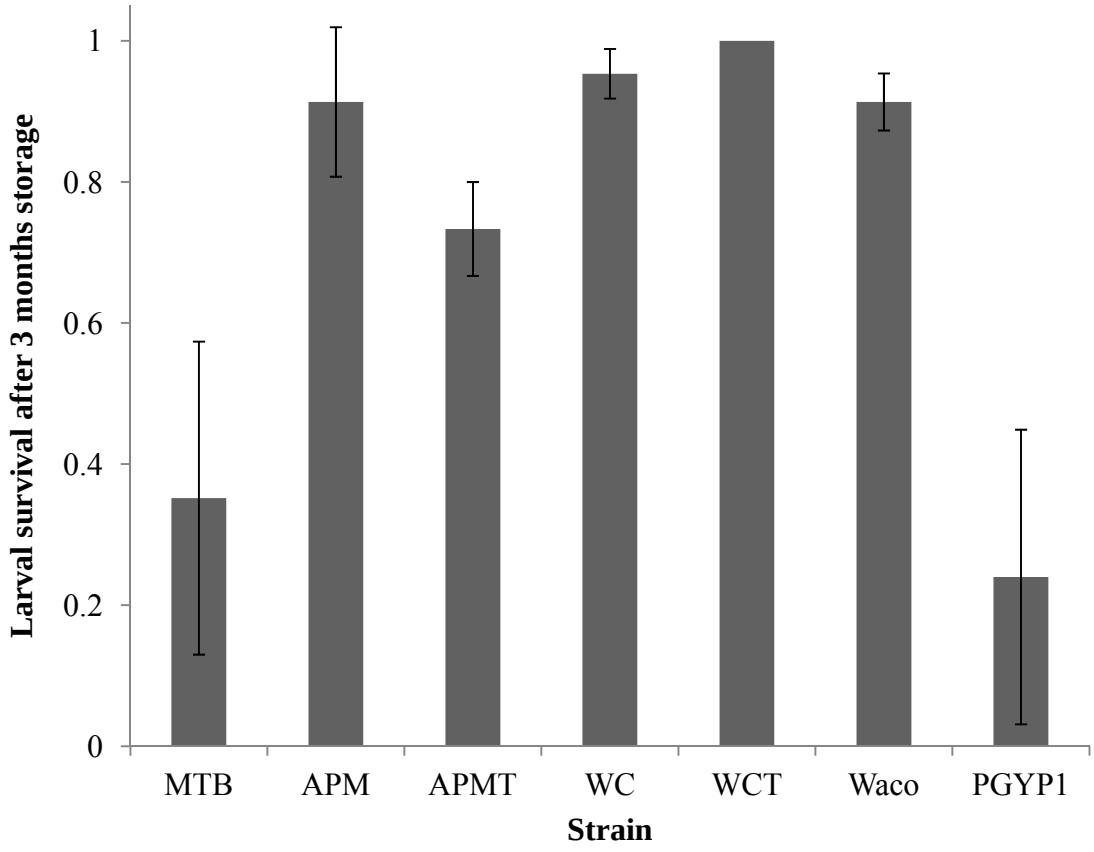


Figure 7.4. Differences in survival of larvae hatched after three months of egg storage (two months for PGYP1). An ANOVA determined that there was a significant difference in larval survival across the seven strains analyzed. Linear contrasts determined that mosquito strains with a microinjected *Wolbachia* infections had significantly lower survival of larvae hatched from egg papers stored for three months. There was no significant difference in larval survival between wild-type strains and tetracycline-treated (aposymbiotic) strains. However, artificial *Wolbachia* infections (*i.e.* strains with a *Wolbachia* infection status not found in nature) also had significantly lower larval survival compared with wild-type mosquitoes.

Chapter Eight

Using a COPAS PLUS machine to characterize mosquito growth during the first larval instar

Introduction

Vector borne diseases still contribute significantly to human deaths worldwide. In 2010 alone, there was an estimated 219 million cases of Malaria worldwide causing an estimated 660,000 deaths (WHO 2012). The cases of Dengue virus, an emerging vector borne disease, has risen exponentially in the last several decades leading to the cause of many deaths, particularly in children (Gubler 2002, Guzman and Kouri 2002). In Italy, a recent Chikungunya virus outbreak has received significant attention (Gratz 2004, Rezza et al. 2007, Bellini et al. 2012). The cryptic nature of many disease vectors highlights the need to understand vector ecology and biology, particularly how the distribution of vectors influences epidemiology.

Medical entomology was largely founded by ecologists of disease vectors (Mullen and Durden 2002). However, the popularization of insecticides in the 1950's lead to a decline in the study of vector ecology (Mullen and Durden 2002). Recent trends show that insecticide resistance is quickly evolving, with some mosquito species demonstrating resistance to all major insecticide modes of action (Vontas et al. 2012). Coupled with globalization and a growing awareness of sustainable practices, there has been a rediscovery of traditional vector biology and ecology studies (Vontas et al. 2010). Particularly, traits and genes are being identified and manipulated to help control vector populations with minimal human input.

Wolbachia pipientis is an intracellular bacterium that is being evaluated as a potential biological control agent (Werren et al. 2008, Hoffmann et al. 2011). *Wolbachia* is an evolutionarily successful bacterium that is estimated to reside in nearly half of all insect species (Zug and Hammerstein 2012). Arthopods infected with *Wolbachia* are subject to reproductive manipulations, which help facilitate the spread of infection into a population (Werren et al. 2008). There are several phenotypes associated with *Wolbachia* (Hornett et al. 2009, Chevalier et al. 2012, Reumer et al. 2012), but cytoplasmic incompatibility, CI, is the most common (Werren 1997). CI occurs when *Wolbachia* infected females mate with males that are uninfected or have a different *Wolbachia* type (Charlat et al. 2001). These "incompatible crosses" produce inviable eggs due to early embryonic arrest (Lassy and Karr 1996).

CI-inducing *Wolbachia* can be used by two biological control strategies. In one strategy termed population suppression, inundative releases of incompatible males can be made into a population (O'Connor et al. 2012). This is an extension of the incompatible insect technique, which aims to sterilize eggs laid by wild-type females by releasing large numbers of incompatible males into a population and thereby reducing population size (Klassen and Curtis 2005). The second technique, called population replacement utilizes the gene drive mechanism, of *Wolbachia* to replace uninfected population with those that are *Wolbachia* infected (Turelli and Hoffmann 1991, Marshall 2009). The second strategy relies on a secondary factor, (*e.g.* a transgene or increased immune response) to help eliminate disease (Sinkins and Gould 2006, Huang et al. 2007).

Important to disease control strategies implementing *Wolbachia* is an in-depth understanding of *Wolbachia* infections and their effects on vector biology/ecology. To

evaluate the efficacy of *Wolbachia*-based vector control, additional details about this symbiosis are needed. In the study here, I have designed an experiment to qualitatively measure early mosquito development, particularly how does *Wolbachia* infection influence growth rates in the first larval instar. I use a macroscopic flow cytometer to collect data on the size of first instar mosquito larvae, and demonstrate that parameters correlate with physical size. Further uses of the machine, *e.g.* sorting and counting of first instar larvae, are also investigated.

Methods

COPAS PLUS general overview

All analysis was performed using a COPAS PLUS machine (Union Biometrica, Holliston, MA). COPAS (complex object parameteric analyzer and sorter) PLUS machines are large-particle flow cytometers that can analyze objects from 40μm to approximately 700μm (COPAS Manual v2.2). Objects pass through a red diode laser (670nm), at which time the machine measures the axial length of the object and the optical density. Specifically, the machine records two numbers: the time of flight (TOF) and the extinction coefficient (EXT). TOF is a value that represents the number of 0.6μs time slices that an object takes to pass through the laser beam focal point. EXT values represent the amount of light blockage measured when an object passes through the focal point of the laser beam. Concurrently, florescence measurements are taken based on light reflectance collected by three photo multiplier tubes (PMTs). The three florescent measurements are green, yellow and red, which are measured at 510nm, 545nm, and

610nm emissions respectively. Every object that passes through the machine is analyzed and all five measurements are recorded and stored in a data file.

The COPAS PLUS machine runs two different modes. A preliminary data analysis mode, "ACQUIRE," that analyzes particles and records data without sorting. Once data are collected, they can then be viewed by the user and gating and sorting criteria can be set. Gating and sorting are two methods by which particles with specific parameter values can be identified and sorted. When this mode is active, the user can create a polygon around data visualized on a two-dimensional dot-plot (*i.e.* the two dimensions are two parameters collected by the COPAS PLUS, for example TOF x EXT). Objects within the polygon will be "gated." Depending on the desired output, all gated particles can be sorted directly, or a subset of gated particles can be chosen for sorting. Each particle read by the machine is assigned a STATUS code, which identifies whether each particle was too small to analyze, gated or sorted. STATUS codes also record COINCIDENCE, which occurs when two objects are analyzed simultaneously and neither is sorted, and identifies when two objects are sorted into the same well. (A table provided by Union Biometrica describing STATUS codes can be found in the Appendix).

For all experiments discussed here, first instar *Aedes albopictus* larvae were used to determine the gating and sorting criteria. Eggs were hatched in distilled, deionized water for two hours, and the larvae were moved to the COPAS PLUS machine for analysis. Larvae were added into the sample cup along with distilled, deionized water, and data was collected by using the ACQUIRE method. Larvae were distinctly larger than other particles, and gating criteria were set to capture all larvae. All gated particles

were sorted. The same program was used over many sorting events and was deemed accurate.

Predictive modeling of larval size

To determine if measurements taken by the COPAS PLUS machine were predictive of larval size, larvae were sorted by the machine and then measured using a light microscope. Eggs were hatched in distilled, deionized water with liver powder. The resulting larvae were taken to the COPAS PLUS machine and sorted into 24-well plates using the sorting criteria defined by the ACQUIRE process mentioned above. The COPAS PLUS dispensed one first instar mosquito larvae into each well. After sorting, additional water was added to each well and larvae were returned to the lab. Larvae were then killed using 70% EtOH, and were removed from each well and placed on a microscope slide. All larvae were viewed with a Leica™ dissecting microscope, and images were taken using a digital camera. Images were then analyzed using the ImageJ software to determine the physical measurements of each larva photographed (Barboriak et al. 2005). A predictive, linear model incorporating all five COPAS PLUS parameters was then developed to predict larval axial length. To generate the coefficients for each factor in the predictive model, the MATLAB function *regress* was used; specifically, physical length was the response variable and each COPAS PLUS measurement was used as a predictor variable. A linear model was then built using those coefficients and was used predict larval size. To verify the predictability of axial length, all measurements were taken as parameters into Equation 8.1.

$$length = -4.39 * 10^{-05}(TOF) + 0.012(Green) + 6.71 * 10^{-4}(Yellow) - 6.46 * 10^{-4}(Red) + 1.75 * 10^{-4}(EXT) + 0.140 \quad (8.1)$$

The predicted axial length was then compared to the measure axial length via a linear regression (statistics were performed using JMP 9.0).

Effect of sorting larvae on adult viability and sexual size dimorphism

To determine if the sorting procedure had any negative effects on larvae, mosquitoes were sorted using the COPAS PLUS machine and then their survival to adulthood was determined. Two strains of *Aedes albopictus* mosquitoes were used to examine sorting efficiency, the cost of sorting on larval viability, and whether sexual size dimorphism is detectable by the COPAS PLUS machine in early larval instars. IH27 is a wild-type *Aedes albopictus* mosquito strain that is naturally superinfected with two different *Wolbachia* infections. UT is an uninfected mosquito strain generated by tetracycline treatment (Dean and Dobson 2004). Larvae were hatched in dilute liver powder solution for approximately four hours. Mosquitoes were then sorted by the COPAS PLUS, using criteria from the ACQUIRE procedure above. Larvae were sorted into a 24-well plate. In each well, additional water was added along with a small amount of liver powder solution to provide enough resources such that larvae would survive to pupation. Larval survival was monitored and adults were allowed to eclose and were identified by sex. Sorting efficiency was determined by calculating the number of wells with a first instar larvae divided by the total number of wells. No statistics were

conducted, instead opting for a qualitative accuracy of sorting. Adult viability was also determined by dividing the number of successful adult eclosions by the total number of wells to obtain a survival proportion. No statistics were performed here, opting for a qualitative understanding of survival. However, a logistic regression was used to determine if sex was predicted by TOF parameter values. All statistical tests were performed in JMP 9.0.

Differences in development rate between two strains of Aedes albopictus

A bulk sorting experiment was designed to determine if there were differences between *Wolbachia*-infected mosquitoes and their tetracycline treated analogs. Two strains of *Aedes albopictus* were used here. WC mosquitoes are wildtype *Ae. albopictus* collected from Lexington, KY. WCT are WC mosquitoes that have been cleared of their *Wolbachia* infections using tetracycline. Eggs were hatched in deoxygenated water for 2, 15 and 24 hours. Larvae were brought to the COPAS PLUS machine and sorted into a petri dish using the sorting criteria previously determined. For each time point, at least 112 individuals were analyzed for both WC and WCT larvae. For this experiment, only TOF was compared between strains. An ANOVA was used to determine if there were significant differences in TOF based on hours after hatching (2, 15, 24) and strain. For larvae 24 hours after hatching, two food levels were introduced. One cohort of larvae received abundant food, while another cohort received no additional food. At least 150 larvae were sorted for each scenario and an ANOVA was used to determine significant differences in TOF. The TOF variable was not normally distributed, but after a log transformation, the data were near normal. ANOVAs are robust to non-normal data,

assuming the distribution of a variable is nearly normal and there are large sample sizes. Due to the large number of samples here and approximate normality, I continued with the ANOVA. Post hoc analyses were conducted using a Student's t-test between each strain within hour, i.e. for each hour, WC and WCT were compared.

An additional experiment was designed to determine the effect of starvation on 24 hour old larvae. In this experiment, larvae were hatched in a dilute liver powder solution for six hours. After six hours, a subset of larvae was transferred to a new rearing container filled with distilled, deionized water. These larvae were then allowed to develop an additional 18 hours. In total, the larvae were allowed to develop 24 hours from their hatching in either food deprived or optimal food conditions. These larvae were sorted using the COPAS PLUS machine using the same protocol as the previous experiment. All statistics were performed using JMP 9.0.

Results

Predictive modeling

Using the MATLAB function *regress*, a data matrix of physical measurement and COPAS PLUS parameter estimates was analyzed to determine the coefficients of variables in a multiple linear regression. The multiple linear equation takes all COPAS PLUS parameters as inputs and estimates physical length. Size could not be predicted accurately when larvae were 2 hours old ($R^2 = 0.23$, Figure 8.1a), but at 24 hours larval size can be predicted based on COPAS PLUS parameters ($R^2 = 0.77$, Figure 8.1b).

Sorting accuracy, effect on survival, and sexual size dimorphism

A preliminary experiment was designed to assess some technical aspects of COPAS PLUS sorting. Here, eight 24-well plates of larvae were collected, each plate having one combination of four time treatments (time after hatching) and two infection treatments (WC, infected or WCT, uninfected). For each plate, the sorting of the COPAS PLUS machine was 100% accurate, *i.e.*, there was one first instar mosquito larvae in each well. Larvae were then fed liver powder and some water was added to each well. Plates were monitored for adult eclosion, and an average of 82.29% of sorted larvae survived to adult eclosion (Table 8.1).

After adult eclosion, a logistic regression was used to determine if TOF was a predictor of sex. TOF was not predictive of sex for any plate (data not shown here). Further, there was no significant difference between male or female TOF detected by an ANOVA ($F_{1,157} = 0.05$, $p = 0.82$).

Development rate

Only the TOF parameter (measure of axial length) was used to assess differences in development between mosquitoes. The EXT parameter has a maximum parameter value of 2048, and older first instar larvae will reach a maximum EXT of 2023. Because this occurs before 24 hours, EXT is uninformative for analysis of first instar larvae.

An ANOVA detected a significant difference in TOF with a full factorial design with factors HOUR (meaning hours post hatching) and INFECTION ($F_{5,1591} = 134.51$, $p < 0.0001$). HOUR was a significant factor ($F_{2,1591} = 290.01$, $p < 0.0001$). STRAIN was not significantly different ($F_{1,1591} = 3.73$, $p = 0.05$). The interaction of STRAIN x HOUR was

significant ($F_{5,1591} = 13.73$, $p < 0.0001$). At two and 15 hours after hatching, there was no significant difference between WC and WCT larvae. At 24 hours after hatching, WC larvae were significantly larger than WCT larvae. (Figure 8.2).

Because larvae at 24 hours post hatching were significantly different based on the presence of *Wolbachia* infection, an additional experiment was designed to determine how larvae would respond to food limited conditions. An ANOVA determined that there was a significant overall difference in TOF ($F_{3,841} = 105.74$, $p < 0.0001$). STRAIN was significantly different ($F_{1,841} = 25.12$, $p < 0.0001$), with infected WC mosquitoes being larger than uninfected WCT mosquitoes (Figure 8.3a). FOOD was also a significant factor ($F_{1,841} = 275.04$, $p < 0.0001$). Larvae that were deprived of food had significantly lower TOF compared to well-fed larvae (Figure 8.3b). The interaction STRAIN x FOOD was not significant ($F_{1,841} = 1.30$, $p = 0.26$).

Discussion

Understanding vector biology is critical to the success of disease control programs. Here, the use of the COPAS PLUS large particle flow cytometer demonstrated that machines like the COPAS PLUS can be used to understand the basic biology of disease vectors. Multiple experiments were conducted, and COPAS PLUS parameters generally tracked larval growth. A predictive model was built based on COPAS parameters and was predictive of physical length. Survival of larvae sorted by the machine was high (>80%) and the machine accurately sorts first instar mosquito larvae (100% sorting accuracy). However, TOF was not predictive of sex, and no data suggested sexual size dimorphism at the first instar. A large sample experiment determined that

TOF increases with larval age and that *Wolbachia* infected larvae had larger TOF values than their uninfected conspecifics at 24 hours after hatching. Further, larvae that were deprived of food had significantly lower TOF values than larvae with access to food.

Wolbachia infections exist in a continuum of symbioses, ranging from beneficial to pathogenic. In *Aedes albopictus*, *Wolbachia* infections are beneficial, increasing egg hatch, fecundity and longevity (Dobson et al. 2002a). Other infections cause negative phenotypes like reduced longevity in *Aedes aegypti* (McMeniman et al. 2009). Here, data suggest that *Wolbachia* may increase development rate in early mosquito larval instars. By increasing development rate, infected larvae will become larger, faster which could result in a competitive advantage against other larvae. Female mosquito larvae demonstrate a contest competition functional response (Gavotte et al. 2009). Reaching larger physical size quicker than uninfected conspecifics could alter population dynamics and the likelihood of population replacement.

The development of mosquito larvae is dependent on the presence of food and growth will stall in the absence of food (Padmanabha et al. 2011). Data from the study presented here suggests that first instar larvae are subject to stalled development in the absence of food. Interestingly, there is no significant difference for the interaction of STRAIN x HOUR. This suggests that food deprivation acts independently of *Wolbachia* infection status, which could influence larval competition. Specifically, larvae in food limited environments may experience the same costs irrespective of *Wolbachia* infection status. Further studies examining larval development across many time points with a variety of competition levels would help determine such relationships.

The COPAS PLUS machine has some attractive uses in understanding first instar mosquito biology. First instar mosquito larvae are very small, making their study difficult. Many studies dismiss differences at the first instar because of the difficulty associated with those larvae (McMeniman and O'Neill 2010). But, the COPAS PLUS provides a mechanism whereby larvae can be studied. There are two important uses for the COPAS. The first, as shown here, is measuring larvae and performing hypothesis driven research to better understand the first larval instar. Secondly, the machine is able to sort larvae with high accuracy. This machine can be used to help calibrate experiments or possibly can be used in mass rearing to estimate the number of individuals released.

Additional studies involving the COPAS PLUS are needed to determine its role in understanding vector biology. The invasion of *Aedes albopictus* into parts of the United States is attributed to differences in competitive ability between those mosquitoes and the previously established *Aedes aegypti* populations (Braks et al. 2004). The COPAS PLUS could determine if competition intensity is different during early development because the machine could sort larvae into 24-well plates and the resulting adults could be identified. Such studies could help understand when competition is most important, *i.e.* is competition more important in early or later development. Further, experiments could determine the type of larval competition exhibited in early development and could quantify competition coefficients between mosquito strains or species. Regardless, the COPAS PLUS machine should be investigated as a potential tool to help disease control programs.

Table 8.1. Sorting accuracy and larval-adult survival for *Aedes albopictus* larvae sorted into 24-well plates using the COPAS PLUS machine. WC are wild-type *Aedes albopictus* that are infected with *Wolbachia*, and WCT are tetracycline treated *Aedes albopictus* with no *Wolbachia* infection. Both WC and WCT larvae were sorted at six hour intervals.

plate	sorting accuracy	larval-adult survival
WC 6-hr	100%	79.2%
WC 12-hr	100%	91.7%
WC 18-hr	100%	95.8%
WC 24-hr	100%	79.2%
WCT 6-hr	100%	70.8%
WCT 12-hr	100%	91.7%
WCT 18-hr	100%	79.2%
WCT 24-hr	100%	70.8%
TOTAL	**100%**	**82.3%**

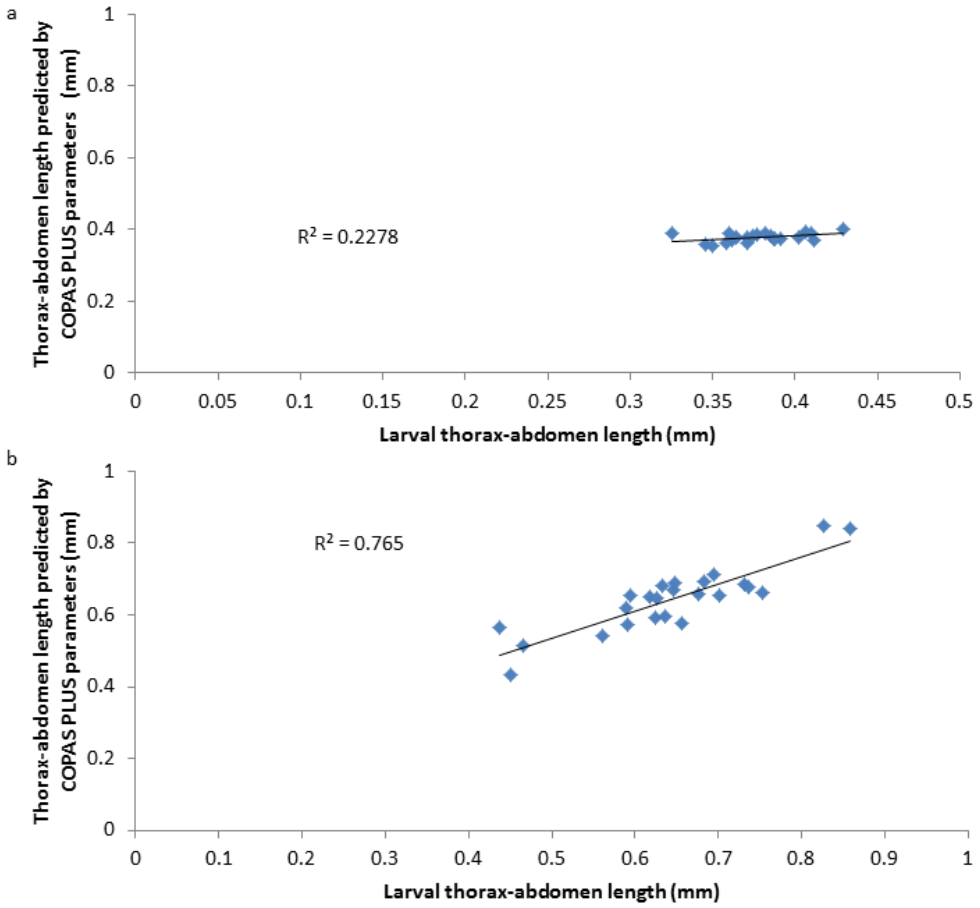


Figure 8.1. Correlations of COPAS PLUS parameters to first instar larval size. (a) Muliple linear regression using COPAS PLUS sorting parameters. By comparing predicted larval side to a physical estimate of size. When larvae are very young, size estimates are not predictive based on the five COPAS parameters. (b) Predicting size with COPAS PLUS parameters. Larvae that have developed for approximately 24 hours can have their size predicted by COPAS PLUS parameters. Predicted values are determined by COPAS PLUS measurements as inputs for Equation 8.1 in the text.

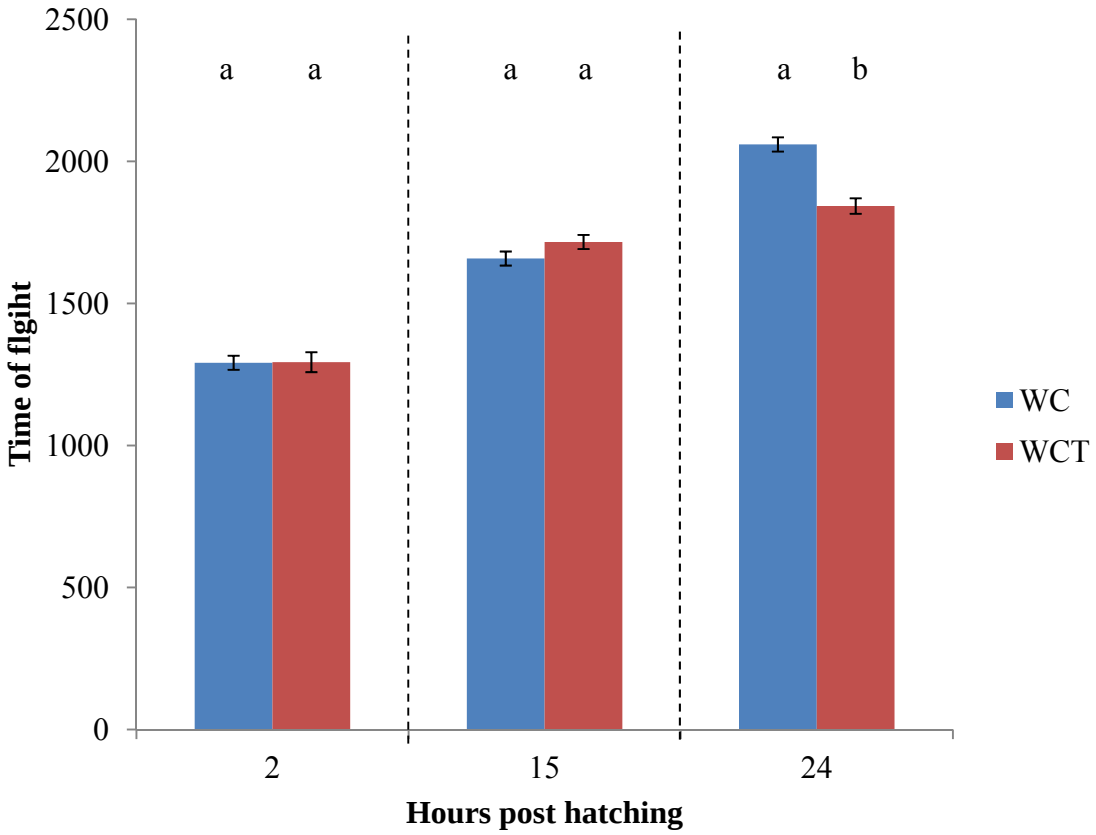


Figure 8.2. Differential development rate as measured by the TOF parameter of the COPAS PLUS machine. TOF is the time of flight parameter which is an approximate measure of axial length. WC are wild-type mosquito larvae that are super-infected with two types of *Wolbachia*. WCT mosquito larvae are aposymbiotic (i.e. has no *Wolbachia* infection). Shown is the mean TOF and error bars are the standard error. Student's t-tests were used to determine significant differences between larvae within each hour. Different letters represent statistically significant differences (Student's t-test $\alpha < 0.05$).

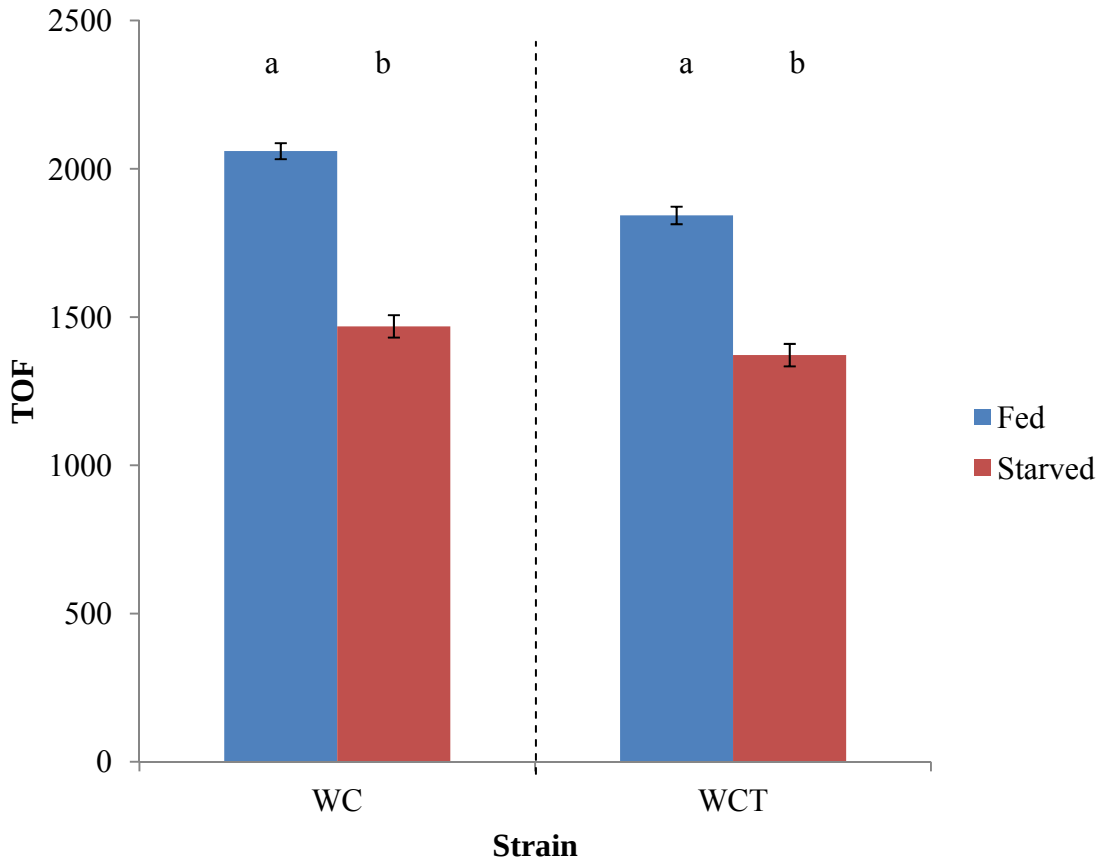


Figure 8.3. Effect of food deprivation on development of 24 hour old larvae. TOF is the time of flight parameter which is an approximate measure of axial length. WC are wild-type mosquito larvae that are super-infected with two types of *Wolbachia*. WCT mosquito larvae are aposymbiotic (i.e. has no *Wolbachia* infection). Larvae were hatched and either fed or starved for 24 hours. No comparisons were made between WC and WCT larvae, but both wild-type (WC) and aposymbiotic larvae (WCT) experience the same significant decrease in TOF when starved. Data shown are the mean TOF values and error bars are standard error. Different letters represent statistically significant differences (Student's t-test $\alpha < 0.05$).

Chapter Nine

Conclusions

In this dissertation, I have described the integration of theoretical and empirical studies to better understand the complex symbiosis of disease vectors and the proposed biological control agent *Wolbachia*. Chapter Two determined that artificial *Wolbachia* infection can decrease larval viability. Increased larval mortality contributed to decreased net reproductive rate. Chapter Three described the development of a simulation model that incorporated *Wolbachia* infection dynamics and insect population dynamics. The model was conceptualized and parameterized based on data collected in Chapter Two. Simulations determined that relative larval viability (*RLV*) is an important parameter dictating population replacement, and that decreases in *RLV* associated with *Wolbachia* infection will inhibit invasion. Chapter Four again examined population replacement, but in the context of field releases that are ongoing in Australia (Hoffmann et al. 2011). The Eliminate Dengue program has released two different mosquito strains into Australia cities. Both strains can provide Dengue interference; however, better disease control strain is associated with high costs of infection (Cyranoski 2012). I modified the simulation model developed in Chapter Three to assess the competition of *Wolbachia* infections with differential costs. High cost infections have low probabilities of population replacement in uninfected populations, and are generally out-competed by less costly infection types. Chapter Five completes the theoretical examination of population replacement by evaluating the likelihood of reverse population replacement, *i.e.* situations where uninfected populations may reinvade infected populations. Infected

populations were typically stable and loss of infection only occurred when costs associated with infection were high. If the level of CI is high, costly infections can be maintained in a population despite the presence of uninfected individuals with higher fitness. Chapter Six was an empirical experiment focused on estimating *RLV* in mosquitoes with artificial *Wolbachia* infections. There was no strong evidence to support a decrease in larval viability due to artificial *Wolbachia* infections. Additional simulations of the model developed in Chapter Three determined that increases in *RLV* could facilitate the invasion of *Wolbachia* into uninfected populations, assuming no other costs of infection. Chapter Seven determined that larval viability is reduced when mosquito eggs are stored or dormant for long periods of time before hatching. Further analyses determined that reduction in viability was not universal and emphasized case-specific *Wolbachia*-host interactions. Finally, Chapter Eight used a large particle flow cytometer to analyze mosquito development within the first larval instar. Infected larvae were significantly larger than uninfected conspecifics, and both infected and uninfected larvae experienced stalled development when deprived of food. All experiments here were designed to work in a larger theoretical framework to help assess the efficacy of *Wolbachia* as a biological control agent.

Many studies have been developed to understand the evolution of *Wolbachia*, the mechanism of CI, and population replacement by CI-inducing *Wolbachia* (Charlat et al. 2001, Tram and Sullivan 2002, Poinsot et al. 2003, Ferree and Sullivan 2006, Bossan et al. 2011). However, studies need to incorporate data from empirical assays regarding changes in pathogen prevalence. *Wolbachia* is known to decrease the presence of several human pathogens in their respective disease vectors (Kambris et al. 2009, Moreira et al.

2009, Kambris et al. 2010, Walker et al. 2011, Andrews et al. 2012, Blagrove et al. 2012, Rances et al. 2012). Despite this new data, little is known about the epidemiological impact of *Wolbachia* on disease prevalence. Theoretical studies combining *Wolbachia* infection dynamics, host population dynamics, and epidemiological models need to be investigated to better evaluate the efficacy of *Wolbachia* as component of a disease control program.

Pathogen interference is associated with the transfer of *Wolbachia* infections to novel insect hosts (Kambris et al. 2009, Pan et al. 2012). Presumably, coevolved infections do not have the same effects on insects as artificial infections since coevolved infections are present in several disease vectors. Notably, a recent Chikungunya outbreak in Italy was vectored by *Aedes albopictus*, which is naturally superinfected with two *Wolbachia* infections (Bellini et al. 2012). One of those infections, *w*AlbB, has been transferred into *Aedes polynesiensis* and results in lower numbers of infective L3 nematodes (Andrews et al. 2012). Similarly, if *Aedes aegypti* is transinfected *w*AlbB, then there is a reduction in Dengue virus titer (Pan et al. 2012). Important to any biological control strategy is an understanding of effective time windows (Labbe et al. 2009). For example, an effective time window may represent the time it takes for resistance to develop for an insecticide, or the time it takes a virus to evolve mechanisms to evade vaccines. Future studies need to focus on evaluating the effective time window for disease control with *Wolbachia*-based approaches.

Currently, no estimates on the long term sustainability of a population replacement *Wolbachia*-based controls strategy are known. Long term, laboratory cage studies could be used to analyze the change in pathogen interference over generation

time. Similarly, studies could assess vector competency in Australia where *Wolbachia*-infected mosquitoes have been established over time. Finally, in vitro assays using cell lines should precede other trials. Studies have shown that costs associated with *Wolbachia* can be reduced by long term serial passage of cell cultures (McMeniman et al. 2008). Similarly, scientists can track pathogen interference over time through these same techniques.

With *Wolbachia* based control strategies being implemented in the field, modeling efforts need to shift from qualitative behavioral understanding to quantitative, predictive applications. There is a large amount of data now available to theoretical biologists and with field trials ongoing, more date will soon be available. Additional studies need to focus on understanding this data and extrapolating results to predict the impact of *Wolbachia* on disease. Statistical models focused on understanding vector population dynamics should be built and analyzed. Theoretical studies should include recent data and need to address the questions mentioned above. With specific areas now being targeted for releases of *Wolbachia* infected mosquitoes, models need to be developed explicitly for those sites and used as tools to increase our understanding of vectored disease epidemiology.

Wolbachia is a promising biological control candidate that has a significant role in integrated pest management programs. The utility of *Wolbachia* is two-fold as either a type of incompatible (sterile) insect technique (O'Connor et al. 2012), or as a gene drive mechanism whereby vector population can be replaced by less competent disease vectors (Hoffmann et al. 2011). Furthermore, there have been many studies focused on *Wolbachia*, providing a rich background of information for scientist and regulators. The

complex nature of *Wolbachia*-host interactions suggests that more studies are needed, but enough of the biology of *Wolbachia* is known to promote its use in disease control programs. *Wolbachia* is an ideal candidate for biological control because it has low levels of horizontal transmission, so its spread to other insects is unlikely (Werren et al. 2008, Watanabe et al. 2012). A major difficulty for many vector control programs is the cryptic habitats of disease vectors, the majority of which need to be treated (Gratz 2004). However, by implementing strategies revolving around *Wolbachia*, scientists can use infected insects to help control all uninfected individuals without seeking out all population sources. Regardless, additional empirical studies are needed to evaluate the efficacy of *Wolbachia*-based control strategies. With the increasing number of studies, a unifying theoretical framework would also be useful that could evaluate the significance of compartmentalized studies. The dissertation presented here demonstrates that integration of empirical and theoretical work can lead to a better understanding of complex biological systems.

Appendix

Chapter 3 Supplementary Material

Wolbachia infections that reduce immature insect survival: Predicted impacts on population replacement

Model Parameters

symbol	definition	initial value	reference
*CI**	level of CI in incompatible crosses (proportion not hatching in incompatible crosses)	0.999	-
*MI**	level of maternal inheritance (proportion of offspring receiving infection)	0.999	-
*RF**	relative fecundity of infected females to uninfected females	0.999	-
*RLV**	relative larval viability of infected larvae to uninfected larvae	0.999	-
*IF**	introduction ratio of gravid infected females to the total adult population at the time of introduction	0.500	-
s	number of larval developmental stages	30	-
u	egg production rate	10.486	derived from equations by Lounibos *et al*. (1985) and Blackmore and Lord (2000)
v	female mass coefficient	3.317	derived from equations by Lounibos *et al*. (1985) and Blackmore and Lord (2000)
w	female mass intercept	0.017	derived from equations by Lounibos *et al*. (1985) and Blackmore and Lord (2000)
z	female mass exponent	0.333	derived from equations by Lounibos *et al*. (1985) and Blackmore and Lord (2000)

symbol	definition	initial value	reference
m_x	theoretical mass of larva at time T	0.005	calculated from laboratory experiment
T_{f0}	female development time at which mass at pupation is $m_x/2$ days	10.480	calculated from data presented in Gavotte *et al*. (2009)
T_{m0}	male development time at which mass at pupation is $m_x/2$ days	8.075	calculated from data presented in Gavotte *et al*. (2009)
μ	baseline larval mortality (i.e. mortality in the absence of age and density dependent effects)	0.100	calculated from data presented in Gavotte *et al*. (2009)
α	density dependent mortality coefficient	0.025	-
β	density dependent mortality exponent	0.061	-
γ	stage-dependent mortality coefficient	1.150	-
ε	stage-dependent mortality exponent	1.200	-
Δt	time step	1.000	-
g	per capita mortality rate of adult females	0.106	Trpis and Hausermann (1986)
j_m	maximum larval male development rate	6.310	calculated from data presented in Gavotte *et al*. (2009)
j_f	maximum larval female development rate	5.469	calculated from data presented in Gavotte *et al*. (2009)
h_m	asymptotic minimum development rate	1.500	calculated from data presented in Gavotte *et al*. (2009)
h_f	asymptotic minimum development rate	1.100	calculated from data presented in Gavotte *et al*. (2009)
q	density-dependent development coefficient	0.035	-
k_m	male growth coefficient	0.153	-
k_f	female growth coefficient	0.174	-
S_E	survival of individuals in the egg stage	1	-

symbol	definition	initial value	reference
S_P	survival of individuals in the pupa stage	1	-
S_M	survival of individuals in the adult male stage (both infected and uninfected)	0.600	Trpis and Hausermann (1986)
H_3	proportion of eggs hatching early	0.200	Gillet *et al.* (1977)
P_B	probability a female mosquito dies before bloodfeeding on host	0.100	Trpis and Hausermann (1986)
P_A	probability a female mosquito dies after bloodfeeding on host	0.100	Trpis and Hausermann (1986)
P_C	probability female changes hosts during bloodfeeding	0.050	Trpis and Hausermann (1986)

Table S1. Glossary of notation, including the initial values for each parameter. In all subsequent model runs, each value remains constant while one key parameter is varied. Key parameters are identified by an asterisk after its symbol.

Extended Appendix

Although this model can be generalized to many holometabolous insect systems, our particular case study involved mosquitoes in the genus *Aedes*. The key functions were specifically designed to address mosquito population dynamics.

$$R = \frac{(j-h)\Delta t e^{-qB} + h\Delta t}{s} \quad (1)$$

The development rate R (developmental stage units) is an exponential function of time-step duration (Δt) and total larval population mass B (units of mass), with an asymptotic minimum that reflects the minimum development rate that larva will develop when larval competition is high. j is the maximum development rate (developmental stage units), h is

the asymptotic minimum development rate (developmental stage units), Δt is the time step (units of time), q is the density-dependent development coefficient (units of (mass)$^{-1}$), and s is the total number of developmental stages. The rate of development increases inversely with larval competition, and the rate is dependent on the overall number of divisions in the larval stage. This function was developed to describe patterns in experimental data presented by Gavotte *et al*. (2009). Default parameter values were derived from curve fitting raw data. Furthermore, a similar functional relationship between immature development time and immature density has been described previously (Barbosa et al. 1972, Peters and Barbosa 1977).

$$S_L = e^{-(\mu+\alpha B^{\beta}+\gamma d^{-\varepsilon})\Delta t} \quad (2)$$

Larval survival, S_L, is an exponential function where the form of the function is governed by the development stage and density of the larvae. μ is the baseline mortality rate of mosquito larvae in the absence of competition (units of (time)$^{-1}$). α is the coefficient controlling density dependent mortality (units of (time)$^{-1}$). B is the total larval biomass (dimensionless), β is the exponent controlling density dependent mortality (dimensionless), γ is the coefficient that decreases mortality as development stage increases (units of (time)$^{-1}$), d is the developmental stage index, ε is the exponent that decreases mortality as development stage increases (dimensionless), and Δt which is the time step (units of time) thus, S_L increases as the stage of larval development increases but will decrease when competition increases. The equation presented here was modified from an equation presented by Dye (1984) to incorporate stage dependent mortality and is similar to other simulation models (Focks et al. 1993b, Magori et al. 2009).

Furthermore, the survival from eggs to pupae was similar to that described by Southwood *et al*. (1972).

$$M = \frac{m_x e^{k(d-1)}}{1 + \frac{1-c}{c}^{\frac{T_0 - T}{T_0}}} \quad (3)$$

This function depicts a reverse sigmoid relationship for mosquito body mass, *M* (units of mass), versus development time. The assumption here is development time is lengthened due to higher levels of intraspecific competition. m_x (units of mass) is the theoretical maximum mass of a given mosquito at time *T*. m_x is linked to *c* (dimensionless), which is the percent of m_x that is attainable. *k* (dimensionless) is the growth coefficient; T_0 (dimensionless) is the development time at which mass at pupation is $m_x/2$ days, and *T* (dimensionless) is development time. *d* (dimensionless) represents the total number of development stages completed by the larval cohort. The values *c* and m_x were derived from measurements taken from first instar larva. A cohort of larvae were dried, and weighed, and the average was taken. The value of *c* was arbitrarily set to 0.999 and m_x was then back calculated. The equation and parameter values presented here were derived to fit data published by Gavotte *et al*. (2009).

$$F_s = e^{-gA} \quad (4)$$

Female survivorship, F_s, is an exponential decline where the survivorship probability decreases when the age of the female increases. *g* is the per capita mortality rate of adult females (units of $(\text{time})^{-1}$) and *A* is the current age of the female (units of time). In

general, over time, the probability that a female will die increases with the age of the female. The equation here is taken directly from Trpis and Hausermann (1986).

$$E = u\Delta t e^{v(M_f+w)^z} \quad (5)$$

Overall egg production, E, is governed by body mass, which is redefined from Lounibos et al. (1985) to mean body mass (which Blackmore and Lord relates to wing length in their equation). u is the egg production rate (units of $(\text{time})^{-1}$); Δt is the time step (units of time); v is the female mass coefficient (units of $(\text{mass})^{-1}$); M_f is the body mass of the ovipositing female (units of mass); w is the female mass intercept (units of mass), and z is the female mass exponent (dimensionless). Female egg production is a composite equation derived by combining two equations that were published previously in Blackmore and Lord (2000) and Lounibos (1985).

Sensitivity analysis

The model presented here was designed to (1) represent the life history of holometabolous insects and (2) clarify how changes to life history and *Wolbachia*-related parameters affect the probability of population replacement by *Wolbachia*. To assess the robustness of this model, a detailed sensitivity analysis was conducted for each parameter defined in Table 1 (excluding s and Δt). s (stage index) can be set to any arbitrary value. The number of stages chosen should reflect the desired detail in larval population dynamics. If larval dynamics is likely not an important factor in overall population dynamics, then the number of stages could be small (approximately the true number of larval instars or less). When larval temporal dynamics and survival are important, more

resolution in the larval stage (i.e. many stages per true larval instar) might provide more accurate results. For each parameter, a measure of sensitivity (MOS) was determined by calculating a ratio of the percent change in the probability of population replacement by *Wolbachia* relative to a ten percent change in the initial parameter value. Parameters were then categorized based on their level of sensitivity into four levels: parameters insensitive to change (MOS ≤ 0.2), parameters moderately sensitive to change ($0.2 >$ MOS ≥ 0.5), parameters strongly sensitive to change ($0.5 >$ MOS ≥ 1.0), and parameters supraproportionally sensitive to change (MOS > 1.0). The probability of population replacement was determined by averaging the number of population replacement events occurring in one hundred simulations per replicate for ten replications.

Table S2 lists each parameter and the corresponding measure of sensitivity. Eight of 28 parameters are demonstrated to be insensitive to change to initial parameter values. These parameters are found in equations dictating development rate (h_m and h_f), oviposition (z), larval survival (β), and mosquito body mass (T_{m0}, and T_{f0}). Also the deterministic male survivorship rate (S_M) and the probability that a female changes host during a bloodmeal (P_C) had no effect on the probability of population replacement predicted by the model.

Nine parameters are shown to be moderately sensitive to changes in the initial parameter value. Of these nine parameters, two affect larval survival (α and μ), two are associated with mortality for bloodfeeding females (P_B and P_A), two determine mosquito body mass (m_x and k_m), and the others affect oviposition, development rate, and the proportion of eggs hatching on day three (w, q, and H_3 respectively).

The remaining eleven parameters altered the probability of population replacement substantially, reaching MOS values exceeding 0.5 and 1.0. Parameters significant to change ($0.5 > MOS \geq 1.0$) included the maximum male development rate j_m, the egg production rate u, the per capita rate of female mortality g, and the stage-dependent exponent ε. Six parameters are supraproportionally sensitive to changes in initial parameter values. These parameters affect female development rate (j_f) and body mass (k_f), oviposition (v), and mosquito survival (S_E, S_P and γ).

The model presented here tracks the invasion and establishment of a *Wolbachia* infection in a population size of approximately 110 adult mosquitoes. Parameters that significantly affect the probability of population replacement by *Wolbachia* also tend to decrease the overall population size at which the model reaches equilibrium. With some parameters, a ten percent decrease in the initial parameter value lowers the adult population size considerably (e.g. when $S_E = 0.9$, the average population size during a simulation of the model decreased to less than 50 individuals). In these scenarios, the initial *Wolbachia* infection frequency (*IF*, Table 1) remains the same, but the absolute number of total female adults released decreases with the total adult population size. The model incorporates stochastic adult female survival dictated by the female survivorship function (Equation 4). Adult females that have matured sufficiently to take bloodmeals and oviposit have a survivorship of approximately 65%. Given very small population sizes and stochastic survivorship, *Wolbachia* infections can fail to invade due to stochastic female death (demographic stochasticity).

Parameters that can decrease adult population size occur in all of the equations defined here; for example, k_f and k_m can increase or decrease the biomass of larvae.

Increasing larval biomass results in decreased larval survival (B, Equation 2) and adult population size. Furthermore, decreases in k_f can decrease the total number of eggs produced by females since body mass is positively correlated with egg production (M_f, Equation 5). Because mosquito size reflects development time, changes to the maximum development rate, j, was sensitive to change. Short mosquito development times reflects ideal rearing conditions (i.e. low larval densities), which increase body mass (T, Equation 4). As discussed, increases or decreases in body mass substantially affect total population size in all lifestages. Increasing or decreasing various parameters in the larval survival equation can have a variety of effects on the population size, but decreasing survival reduces population size and population replacement in the example shown (Table S2). Similarly, changing parameters that resulted in lower fecundity (Equation 5) tended to inhibit invasion by *Wolbachia*. Increasing the per capita mortality rate for adult females (g, Equation 4) decreases the number of females that survive to produce eggs, decreasing population size and population replacement.

The conclusions from the sensitivity analysis were similar to conclusions described in the main manuscript. In general, the survival of immature lifestages influences the adult population size. As shown above, the number of adults can affect the invasion and establishment of *Wolbachia* by genetic drift when the population size is small. Because these data are typically unavailable, the sensitivity of the model to these parameters underscores the importance of additional empirical work. Also parameters affecting adult female individuals (particularly those which ultimately affect fecundity) are very important to the model's quantitative and qualitative behavior. We therefore emphasize that studies examining the spread of *Wolbachia* should characterize host

population dynamics as well as possible, since these features so strongly influence the invasion and establishment of *Wolbachia*.

parameter	measure of sensitivity
S_M	0.03
z	0.06
P_C	0.09
h_f	0.10
β	0.12
T_{m0}	0.13
T_{f0}	0.15
h_m	0.16
m_x	0.21
H_3	0.21
w	0.24
P_B	0.34
α	0.35
P_A	0.36
μ	0.40
q	0.42
k_m	0.46
c	0.54
j_m	0.58
u	0.67
g	0.72
ε	0.85
S_P	1.16
γ	1.44
j_f	1.52
v	2.56
k_f	4.03
S_E	4.72

Table S2. The measure of sensitivity (MOS) for all parameters defined in Table 1. MOS was calculated by increasing or decreasing each parameter by 10% then calculating the

rate of change in the probability of population replacement. The parameters were categorized based on the magnitude of their MOS, ranging from insensitive to change (MOS ≤ 0.2), moderately sensitive to change (0.2 > MOS ≥ 0.5), strongly sensitive to change (0.5 > MOS ≥ 1.0), and supraproportionally sensitive to change (MOS > 1.0).

COPAS PLUS STATUS codes

‘5’ - acquired normally

‘6’ - dispensed normally

‘7’ - same drop coincidence

‘8’ - previous event coincidence (coincidence ON)

‘10’ - extra pulse at conversion time coincidence

‘11’ - stretched drop (coincidence OFF, zero separation)

‘12’ - TOF greater than drop size

‘13’ – dispensed, ignored coincidence state (coincidence OFF)

‘16’ - outside region

‘22’ - incomplete signal conversion , or TOF < minTOF

‘23’ - TOF Limits exceeded

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Publications

- **Crain PR**, Crowley PH, Dobson SL. 2012. ***Wolbachia* re-Replacement without Incompatibility: Potential for Intended and Unintended Consequences.** (Submitted to *Journal of Medical Entomology*).

- Mains JW, Brelsfoard CL, **Crain PR**, Huang Y, Dobson SL. 2012. **Population impacts of *Wolbachia* on *Aedes albopictus*.** *Ecological Applications*

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