WOLBACHIA-MEDIATED POPULATION REPLACEMENT IN DENGUE MOSQUITO VECTORS

By

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ABSTRACT

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There are many vector-borne diseases, including dengue, that lack vaccines or effective treatment options, resulting in vector control being the primary disease control strategy. *Wolbachia*, an intracellular bacterium that can spread itself through a population via cytoplasmic incompatibility (CI), has been shown to inhibit the transmission of a number of the deadly human pathogens, like dengue and *Plasmodium,* in mosquitoes. In order to utilize *Wolbachia* to make mosquitoes inhospitable to the pathogens, we have to create a more efficient population replacement strategy such that disease transmission can be interrupted completely and rapidly. In this work, we performed *Aedes aegypti* laboratory cage studies in which the *Wolbachia*-infected females were released once, at the beginning, followed by continued inundative infected male release at every generation. We found that this inundative male release could accelerate the process of population replacement. We also designed a new mathematical model that is capable of accurately predicting the generation in which population replacement will occur. To develop a population replacement strategy for *Aedes albopictus* we introduced the third type of *Wolbachia*, *w*Pip, into this mosquito species to create a transinfected line carrying a triple *Wolbachia* infection. We characterized the pattern of CI induced by this novel artificial infection through crossing assays. We found that the triply infected *Ae. albopictus* induces unidirectional CI when crossed with the wild type doubly infected mosquitoes, supporting its potential to be used in population replacement study.

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Chapter 1

An introduction to *Wolbachia*, dengue, cytoplasmic incompatibility, and population replacement

Insect-borne diseases are the leading causes of illness and death in the world today (Iturbe-Ormaetxe et al 2011). The case numbers increase with the expansion of globalization and human travel. Even with inspection protocols in place, it is impossible to catch every single non-native and possible pathogen carrying insect as it enters the country. This leaves every country that imports and exports goods, or has an international travel industry vulnerable to exotic vector-borne diseases. Particularly dangerous are those diseases, such as dengue virus, that have no vaccine and no treatment options.

Approximately 2.5 billion people are living in areas at risk for dengue infection and there are at least 50-100 million cases of dengue fever and 500,000 cases of dengue hemorrhagic fever (DHF) reported each year (Gubler and Clark 1995; WHO 2009). However, even these numbers are conservative due to the probability of a large number of cases remaining unreported and the limited availability of data for those that are reported (Gubler 1998). Reports of DHF have increased by an average of 5-fold on a global scale, with the majority in Asia (Gubler 2002). Due to a lack of vaccines and treatment options, vector control is a primary dengue control strategy.

Dengue virus, with four serotypes that are all transmittable to humans, can infect all ages and races of people. The common symptoms of dengue fever are mostly similar to the influenza virus, including a high fever accompanied by some of the following: chills, pain, nausea, vomiting, headache, and a rash (Cobra et al 1995, WHO 2012). The symptoms usually last for approximately one week and begin between 4-10 days after bitten by the infected mosquito

(World Health Organization 2012). The disease becomes fatal when it develops into dengue hemorrhagic fever. This form of the disease has symptoms of nausea, vomiting, headache, abdominal pain, a fever that lasts 2-7 day, easily bruising, bleeding from the gums and nostrils, skin hemorrhaging, and possible internal bleeding (CDC 2012). Dengue Shock Syndrome (DSS), in addition to the symptoms of dengue fever and dengue hemorrhagic fever, causes circulatory failure and death (WHO 2009).

Ae. aegypti and *Ae. albopictus* are two important disease vectors for dengue virus. *Ae. aegypti*, also known as the Yellow Fever Mosquito, is the primary vector of dengue fever and dengue hemorrhagic fever. This mosquito originated in Africa and likely began to spread through the world via the slave trade and it continued to spread through various trading routes and wars (Mousson et al. 2005). *Ae. aegypti* is very well adapted to living in urban areas. They prefer stagnant water sources for their larvae, so man-made containers (like buckets, tires, etc.) are perfect habitats for them. As of 2001, *Ae. aegypti* has spread across parts of Asia, Africa, South America and North America (Gubler 2002). It is now found in tropical areas around the world (Mousson et al. 2005).

Ae. albopictus, also known as the Tiger Mosquito, is another mosquito species that has readily adapted to urban habitats. This species can breed in both man-made and natural containers (Knudsen 1995). It originated in South-East Asia and spread through the world via trade routes and human travel (Mousson 2005). Until now, this mosquito species has been found on six of the seven continents and is rapidly spreading; Antarctica is the only continent lacking the species due to climate conditions (Paupy et al 2009).

Both societal and environmental stumbling blocks have caused issues with the implementation of many vector control strategies. In the case of pesticides to control the vector population, there are increasing problems in development of resistance (Lenormand et al. 1999; Chareonviriyahpap et al. 1999; Rose 2001), possible negative effects on non-targeted species, and potential toxicity to vertebrates. These have been observed for the insecticides including Temephos, Methoprene, Permethrin, DDT, etc. (Rose 2001). There are several problems with the sterile male release strategy. It is not self-sustainable and it is work-intensive, as the males must be released every generation or the natural population will be able to build up again. In addition, it is not always effective. It is possible for some males that are released to be fertile or to be too weak to properly mate with the females. Transgenic mosquito technology is facing challenges of containment, risk management, regulation approval, fitness costs (Irvin et al. 2004; Sperança and Capurro 2007) and the capability of the transgene to spread itself through a population (Crampton et al. 1990; Scott et al. 2002). These issues cause many of these options to be impractical for long-term use (Kyle and Harris 2008).

Despite the problems associated with many of the control strategies, each approach has its unique advantage compared to the others, resulting in a possibility to develop an integrated vector control strategy. Pesticides, for example, are generally fast acting and, while slower, with proper application sterile males can be an effective method of control for disease vectors (Esteva and Yang 2005). Transgenic mosquitoes that are altered to be resistant to the diseases they transmit have a great deal of potential use in disease control projects (Marshall and Taylor 2009). Release of incompatible males for population suppression has been shown to be effective in eliminating a population (Laven 1967). *Wolbachia*–mediated population replacement, due to its

self-sustaining nature, use of naturally occurring bacteria, and use of the native species, can be considered a far more appealing option for vector control strategies.

Wolbachia is a genus of obligate reproductive endosymbiotic bacterium related to *Rickettsia* (Werren and Windsor 2000). Found in all of the major insect orders (Werren and Windsor 2000), *Wolbachia* is estimated to be present in approximately 66% of insect species (Hilgenboecker et al. 2008). *Wolbachia* can be transmitted vertically, from mother to offspring. The bacterium is associated with cytoplasmic incompatibility (CI), parthenogenesis, feminization of males, decrease in adult life span, and male killing (Iturbe-Ormaetxe et al 2011, Werren and Windsor 2000; Werren 1997). Recently, *Wolbachia* was found to inhibit the transmission of several types of human pathogens within the vector (Moreira et al. 2009; Bian et al. 2010)

Wolbachia has great potential in the control of dengue because it can spread into mosquito population through CI and cause the mosquitoes to be refractory against dengue virus. In the CI cross, a *Wolbachia* infected male mates with a female that is uninfected or one lacking the same strain of *Wolbachia*, resulting in few to no viable offspring (Jansen et al. 2008; Hoffmann et al. 1990). We do not fully understand the mechanism by which CI occurs, but one model to explain it is that *Wolbachia* function through a modify/rescue system. In this model, the sperms are modified by *Wolbachia* in the infected males such that they cannot fertilize the eggs, unless the same type of *Wolbachia* presents in the eggs to rescue this sperm modification (Zabalou et al. 2008, Dobson 2004). In a simple unidirectional CI, *Wolbachia* provides advantage to infected females such that it can invade into the uninfected population (**Figure** 1.1). An example of this is seen in the single infected *Ae. aegypti* line generated via microinjection (Xi et al. 2005b).

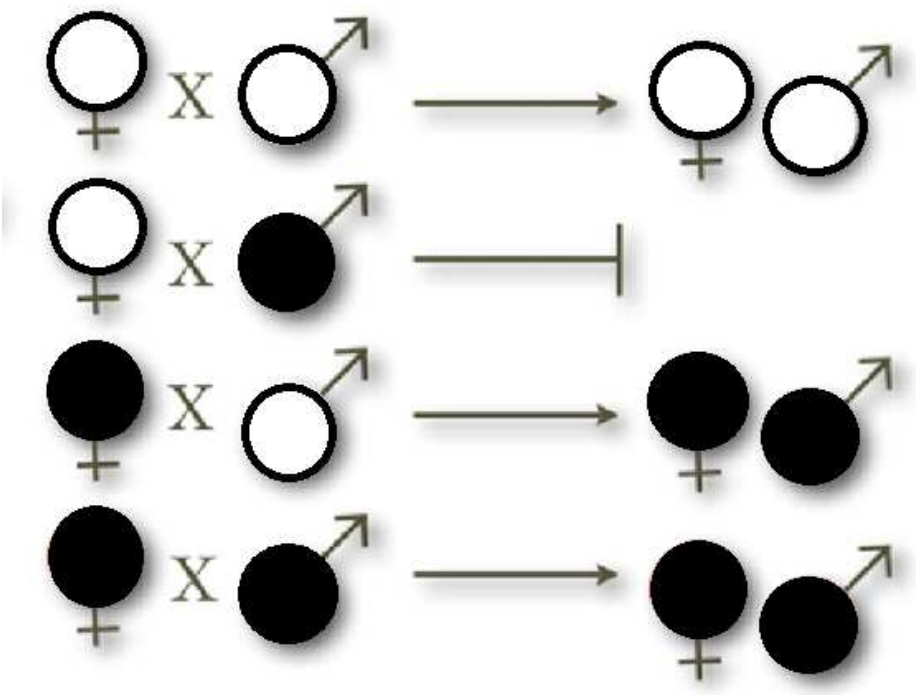

Figure 1.1: A Unidirectional Cytoplasmic Incompatibility occurs when *Wolbachia*-infected individuals (Solid symbols) cross with the uninfected individuals (Empty symbols) (Brelsfoard & Dobson 2009).

It gets more complex when the host is infected with two or more *Wolbachia* types. This can lead to bidirectional CI and additive unidirectional CI. Bidirectional CI and additive CI are types of CI involving at least two different *Wolbachia* infections. In bidirectional CI the population has some individuals infected with one type of *Wolbachia* and some individuals that are infected with another type of *Wolbachia* (**Figure** 1.2). This type of CI results in approximately a fifty percent chance of compatible matings and a fifty percent chance of incompatible matings in the population. Additive CI occurs when it is possible to have individuals that are infected with two or more *Wolbachia* types at the same time and within the

same mosquito (**Figure** 1.3). (Dobson Lab Web site (citation))

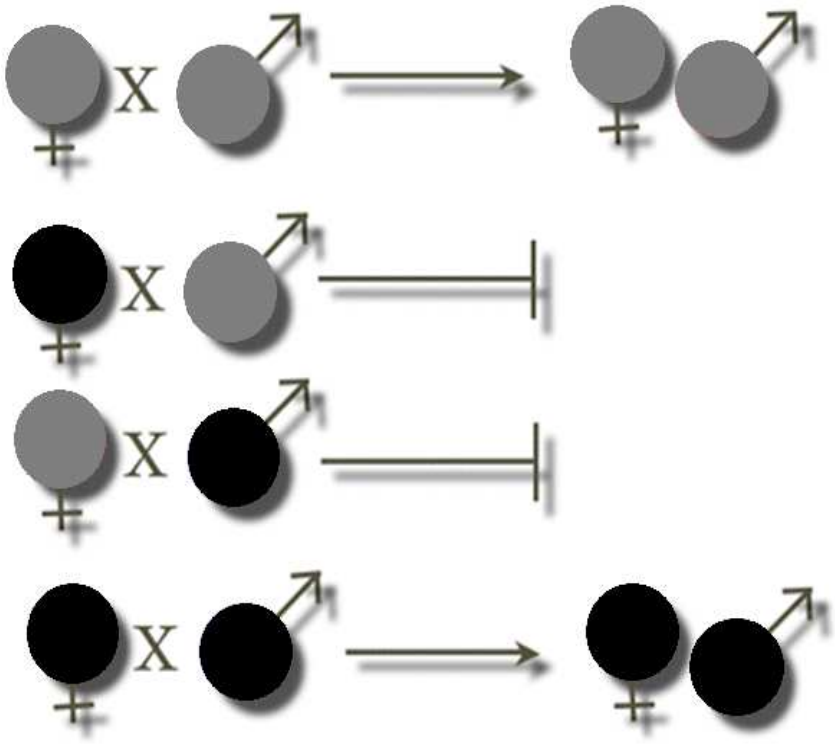

Figure 1.2: Bidirectional CI occurs when a population is infected by two types of *Wolbachia*. Grey: type 1, Black: type 2 (Brelsfoard & Dobson 2009).

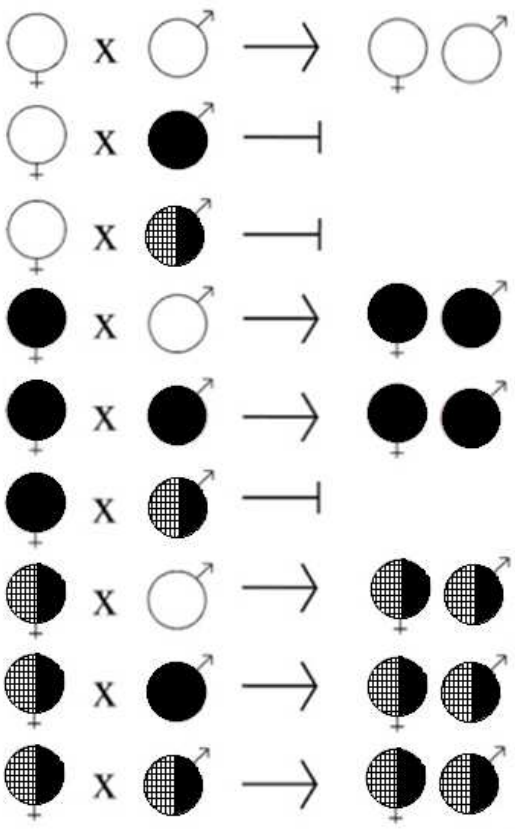

Figure 1.3: An additive CI occurs in the crosses between superinfected and single infected lines. Solid black: single infection, Solid white: no infection, 1/2 solid and 1/2 patterned: 2 types of infection (Brelsfoard & Dobson 2009; Dobson, Additive Incompatibility).

Using *Wolbachia* infected males to induce CI is a possible alternative to the sterile insect technique (SIT), also referred to as population suppression or Incompatible Inset Technique (IIT). SIT is often induced by irradiating males to kill the sperm which results in a lack of viable offspring (White, Rohani, and Sait 2010; Weeks, Turelli, Harcombe, Reynolds, Hoffmann 2007). However, irradiation can have serious fitness cost for the treated insects. This includes increased mortality and decreased longevity, greatly lowering the probability of mating success. SIT can also result in other fitness costs and incomplete sterility (Helinski, Parker, and Knols 2009). As

another advantage over the sterile male technique, the *Wolbachia* infection persists throughout generations, and is thus a self-sustaining alternative to repeated use strategies.

Wolbachia can convey a resistance to several different diseases, including dengue virus in its mosquito hosts (Hedges 2008; Mousson et al. 2012; Teixeira et al. 2008; Moreira et al. 2009; Bian et al. 2010). By conveying this resistance it is possible to block dengue transmission to humans. Thus, the various types of *Wolbachia* are viable options for disease control if they can be established within a population. The bacterium, despite not wiping out or greatly reducing the mosquito population in the release area like the sterile male technique or IIT, still reduces disease transmission. A method for using CI to drive *Wolbachia* into a population is referred to as population replacement. Population replacement is where a naturally disease susceptible population is replaced by a population that is resistant to pathogens. Population replacement studies in *Ae. aegypti* benefit from the fact that the wild population is not infected with *Wolbachia* and thus avoid a need of complicated system. Establishment of a stable single *Wolbachia* infection allows CI to take place in the pattern shown in **Figure** 1.1. There is approximately a 50% chance that the offspring will be infected with *Wolbachia,* a 25% chance that the offspring will be uninfected, and a 25% chance of CI occurring and causing the embryo to fail to form. In the case of *Ae. albopictus*, the system is much more complicated because the two types of *Wolbachia, w*AlbA and *w*AlbB, are present in the natural populations. For CI or population replacement to occur this line requires either a third *Wolbachia* type, such as *wPip* which is derived from *Culex pipiens* mosquitoes (Hertig 1936), to be added or the current *Wolbachia* types to be cleaned from the mosquito line and replaced with a novel infection type. Population replacement has been shown to be possible in both laboratory studies (Xi et al. 2005b) and in field studies (Turelli and Hoffmann 1991 and Hoffmann et al. 2011). This is

extremely important due to the disease limiting capabilities of *Wolbachia*. As mentioned earlier, the transmission of dengue virus can be limited and, potentially stopped, by *Wolbachia*. The key is how to effectively drive the bacterium into a target population and establish a permanent and stable infection.

Typically embryonic microinjection is used to establish a novel *Wolbachia*-host association (Xi et al. 2005a). At an early embryonic development stage, *Wolbachia* is transferred from the infected donors into the posterior area of recipients, followed by intensive screening by PCR to identify a line with a stable symbiosis formed. A number of stable infected lines carrying different infection types have been generated using this method, including *Ae. aegypti* with *w*AlbB (Xi et al. 2005b), *w*MelPop (McMeniman et al. 2009), and *w*Mel (Walker et al 2011), *Anopheles stephensi* with *w*AlbB (Bian et al. 2013), *Ae. albopictus* with *w*Ri (Fu et al. 2010 and Xi et al. 2006), *w*Pip (Calvitti et al. 2010), and *w*Mel (Blagrove et al. 2012). All these transinfected lines maintain perfect or nearly perfect maternal transmission, and induce strong CI when crossed with individuals that are either uninfected or infected by a different type of *Wolbachia* (Xi et al. 2005b; McMeniman et al. 2009; Walker et al 2011; Bian et al. 2013; Fu et al. 2010; Xi et al. 2006; Calvitti et al. 2010; Blagrove et al. 2012).

There were two main focuses in my research. The first focus of my thesis studies was to determine the optimum conditions necessary for an effective population replacement in *Ae. aegypti*. The second focus was to determine the potential of a novel *Wolbachia* infection to invade into *Ae. albopictus*.

Chapter 2

Increasing the rate of population replacement in *Ae. aegypti* mosquitoes

Introduction

Ae. aegypti (L.) (Diptera: Culicidae) mosquito is a primary vector of dengue virus in tropical regions. There have been some moderately successful attempts at controlling *Ae. aegypti* and innovative approaches continue to be developed. One method to control *Ae. aegypti* populations is the elimination of its habitat, particularly in urban areas. Eggs often hatch in stagnant water where there are many artificial water sources (e.g. buckets, tarps with water pools, etc.), a reduction or elimination of these habitats can significantly decrease *Ae. aegypti density*(Chan et al. 1971). Another common approach to control this mosquito species is ultra-low volume (ULV) application of insecticides (Gubler and Clark 1996). It is well known that, while providing partial control of these pests, insecticides can cause the target organisms to development resistance. This makes future control of the pest far more difficult. The use of microorganisms, such as reproductively disruptive bacteria, has become a way to assist in preventing the mosquito from transmitting the disease. Specifically, large efforts have made to develop bacteria within the genus *Wolbachia* to control vector-borne diseases through population replacement, such that disease susceptible mosquitoes could be modified to become disease resistant individuals (Xi et al. 2005 a & b; Iturbe-Ormaetxe et al 2011).

In previous studies the replacement strategies require the release of a number of infected females that equates to at least 20% of the entire population (Xi et al. 2005b). How long it takes for *Wolbachia* to spread into the populations partially dependent on the size of the overall population (Jansen et al. 2008) and the number of infected individuals that are released. For example, with a 20% release of *Wolbachia* infected females (calculated based on the number

infected females released compared to the total number of females released into a cage), population replacement takes seven generations (Xi et al. 2005b). However, when the initial female release frequency is below a certain threshold, population replacement is likely to fail (Xi et al. 2005b).

The objective of the first study was to increase the rate of population replacement. If the replacement can be accelerated, then the use of *Wolbachia* as a method to control the spread of certain vector-borne diseases will be more effective in field applications. The hypothesis is that additional release of *Wolbachia*-infected males during the spread of *Wolbachia* into a population will accelerate population replacement to occur.

Materials and Methods

Rearing Practices

We used the Waco line of mosquitoes (*Ae. aegypti)*, originating from Texas, for our experiments. The *Wolbachia* infected line of mosquitoes (referred to hereafter as WB1) was generated at the University of Kentucky in 2005 (Xi et al. 2005b). The cages used in this study are made of a light weight aluminum frame (Size 12x12x12" W:H:L Bioquip, Rancho Dominguez, CA, USA) and with hard mesh covering four sides, while the front of the cage is covered in a cloth netting that can be used to seal the cage. The top of the cage can be opened and closed, and is held closed with two metal latches.

All mosquito eggs were stored at a constant temperature and moisture before hatching. The wild type (Waco) and infected type (WB1) eggs were stored for no more than six and three months, respectively because storage for a longer amount of time may result in a dramatic decline in the hatch rate. The eggs were laid on egg paper over the course of three days. The egg papers are pieces of water absorbent brown paper that was cut, to fit the egg cups, is from a

9x11” sheet (Norton, USA). Three egg papers were placed into a 5 ½ oz plastic soufflé cup (Solo Cup co, Lake Forest, IL, USA) and then moistened with water.

The eggs were hatched in a 6% liver powder (Now Foods, Bloomingdale, IL, USA) and water mixture that had been fermented for at least one week to create deoxygenated water and then was diluted to between 30% and 50% with purified water. The water mixture and the egg papers were left in the water overnight in a plastic hatch tray (8.2”x8.34”x2”, 49oz, Pactiv Foodservice/Food Packaging, Lake Forest, IL, USA) and the emerging larvae were split into new hatch trays into groups of approximately 100 larvae per tray. Fresh water was added to dilute the deoxygenated water further. The larvae were fed a 6% liver powder water mixture each day. After 5-6 days the larvae pupated and were collected from the pans for use in the experiment.

Blind tests

Two blind tests were conducted to assess possible technical errors. The first blind test was used to check for and reduce sampling biases in the study. In this experiment, a number of infected and uninfected females were released into a series of cages, each with a different infection frequency. The releasing was done by one other member in the lab. Ten samples were collected in one day in the first test. In the second test, the sample size was increased to 20 samples collected over the course of three days.

The second blind test was run to assess efficacy of PCR assay used to diagnosis *Wolbachia* in this study, (**Table 2.3**). Fifteen *Ae. aegypti* were released into each of three cages. All of the mosquitoes were released by another lab member. All fifteen mosquitoes were dissected and then assessed for infection using PCR.

Population cages setup

Once the larvae pupated, they were separated by sex and placed into individual test tubes until they emerged. In the parental generation (F0), approximately 350 male Waco mosquitoes, 350 female Waco mosquitoes, 90 female WB1 mosquitoes, and 500 male WB1 mosquitoes were placed into individual test tubes. At the onset of the experiment six cages were used, one of which was used for mating between WB1 males and WB1 females. In the mating cage, 70-80 WB1 females and males were released and left alone to mate for two days. The cage was blood-fed with live mice and thirteen blood-fed satiated females (N=52) were selected randomly for release into each of four of the five experimental cages.

In five experimental cages, male mosquitoes were released first to allow both Waco and WB1 males an equal opportunity to mate with the females. Once the males were released, both the infected and uninfected females were released into the cages. They were allowed to mate until they reached 7-10 d post emergence, and they were subsequently blood-fed on live mice. After the blood meal the mosquitoes were given two days to rest for oogenesis, then two 5 ½ oz cups were placed into each cage to collect eggs. Once a sufficient number of eggs (approximately 1000) were laid, which occurred five days after the blood meal, the egg cups were removed and incubated for four days in an environmental chamber in which all of the live mosquito cages were maintained at an average temperature of 26.9C and humidity of 70%. After incubation, the eggs were stored in the environmental chamber in a plastic bag with a moist paper towel.

Once the next generation began, the same hatching procedures as previously described were followed. After the hatching was completed, the number of hatched, un-hatched, and broken eggs on the egg papers for each experimental generation and WB1 were counted. The egg

numbers were then used to calculate the hatch rate for each set of eggs and this procedure was repeated for each generation.

Population Replacement experimental design

There were a total five cages used in this experiment. The cages were set up with varying numbers of *Wolbachia* infected males, with the exception of negative control (**Table 2.1**). This experiment used a system of single *Wolbachia* infected female release in generation zero (F0) and continued inundative release of infected males. For each generation, once the eggs were collected ten adult females were selected at random, dissected, and their ovaries were collected for use in PCR.

Table 2.1: Experimental design of population replacement in the laboratory cages. The Waco line represents the uninfected line and the WB1 line represents the infected line.

Waco♂: WB1♂	No. of Waco ♀	No. of Waco ♂	No. of WB1 ♀	No. of WB1 ♂	Predicted replacement generation
1:0	50	50	13	0	F7
1:1	50	50	13	50	F5
1:2	50	50	13	100	F4
1:4	50	50	13	200	F3
Negative control	50	50	0	0	NA
Total	250	250	52	350	

Polymerase Chain Reactions

DNA Extraction and Incubation

Prior to dissection, 10-20 1.5mL lidded tubes for each experimental set were prepared. Each tube contained 50µL Sodium Chloride-Tris-EDTA (STE) Buffer. One pair of ovaries was placed into each tube. Each sample was then ground, using either a battery operated mortar and pestle, for 90 seconds, or an electrically operated homogenizer (if the homogenizer was used, 100 µL STE buffer and homogenization beads were placed in the bottom of each tube instead of 50 µL). After grinding, 2µL of proteinase K (Roche, USA) was added to each sample and the sample was vortexed and centrifuged. If a pellet had formed after centrifugation, the pellet was re-suspended and the samples were placed in a 55 degree Celsius incubation plate for 1h followed by 15min in a 97 degree Celsius incubation plate to deactivate the proteinase K.

PCR assay

Once the DNA extraction was complete, the samples were kept on ice while 8-strip PCR tubes were prepared for use in the thermo-cycler. Into each tube I placed 22.5 µL of Platinum PCR Supermix (Invitrogen Inc., Grand Island, NY, USA), 0.2 µL of the 81F (forward) and 691R (reverse) *Wolbachia* Surface Protein (WSP) primers (Zhou et al. 1998) and 2.1µL of the DNA sample. The tubes were placed in the thermocycler and a program that heats the machine to 95°C for five minutes, then there were 40 repetitions of spending 30s at 95 °C (melting), 30s at 55 °C (annealing), and 45s at 72 °C (elongation), followed by 7min at 82°C, and then idling at 4 °C. The program ran through 40 cycles to produce the DNA copies.

Once the PCR products obtained, they were mixed with 3µL of loading dye (Invitrogen, CA, USA). Each well of the 2% agrose gel (lifetech, USA) was loaded with 5-10 µL of the sample. Electricity was run through the gel for 25min at 180V and 1A in the case of larger gels

and 12-15min at 150V in the case of smaller sized gels. Once the gel was run it was stained for 15min with an ethidium bromide (10mg/ ml, Sigma, USA) and water mixture. The gel was imaged with UV light using Kodak Molecular Imaging System software (standard edition) in combination with a specialized camera (EL Logic 100 Imaging System) and the results were compared to the 100Kb ladder (Invitrogen) that was prepared by mixing 1µL of the ladder, 7µL of DNAse/RNAse free water and 2µL of loading dye and loaded at the same time as the sample. The WSP shows up as a band that is approximately 610 base pairs long.

Mathematical modeling

In our research, we employ a method of increasing the rate of population replacement in such a way that it can take place in two to six generations as opposed to the current minimum of seven (Xi et al. 2005b). The generations for Population replacement 1 were predicted using the equation that follows:

$a_n = 50(1-p_n)$, $A_n = 50p_n + 50m$, $b_n = 50(1-p_n)$, $B_n = 50p_n$. When n>0. (Eq 2.1)

Where a_n is the number of *uninfected males,* A_n is the *infected males,* b_n is the *uninfected females,* and B_n *infected females.* The number of uninfected females in the next generation G_{n+1} corresponds to the matching frequency between the number of uninfected male and female mosquitoes, and therefore corresponds to $a_n b_n = [50(1-p_n)]^2$. The number of infected females also corresponds to the matching frequency of infected male and female mosquitoes and then is multiplied by the infection cost, $\xi(a_n + A_n) \times B_n = 2500(1+m)p_n\xi$. Together, these numbers define the total infection frequency for G_{n+1}, by calculating:

$$p_{n+1} = \frac{2500(1+m)p_n\xi}{2500(1+m)p_n\xi + [50(1-p_n)]^2} = \frac{(1+m)p_n\xi}{(1+m)p_n\xi + (1-p_n)^2}$$ for all generations n > 0. (Eq 2.2)

To calculate the infection frequency for generation 0 to generation 1, it is necessary insert the actual number of males and females into each of the equations: $a_n = 50(1-p_n)$, $A_n = 50p_n + 50m$, $b_n = 50(1-p_n)$, $B_n = 50p_n$, meaning $a_n = 50$, $A_n = 50m$, $b_n = 50$, and $B_n = 13$. This results in the equation:

$$p_1 = \frac{13(1+m)\xi}{13(1+m)\xi + 50}$$ (Eq 2.3)

For each given fitness cost ξ, and ratio number of males m the infection frequency p_{n+1} is determined by the infection frequency p_n in the previous generation. This equation follows the assumptions that 1) there is an equal chance that any female will mate with any male, she will not discriminate based on infection presence or absence, 2) there is equal sex determination for each experimental generation, the probability that any one egg will be male or female is .5, 3) there is complete CI sterility, all incompatible matings will result in no offspring, and 4) 100% maternal transmission, all offspring of infected females will have the same infection type as the female. This model is designed to take into account a varying number of infected males being released into the population.

The equation that was designed follows the properties of uniqueness and monotonicity. So, for each given fitness cost ξ, the infection frequency p_{n+1} is *uniquely* determined by the infection frequency p_n in the preceding generation; and the sequence p_n is increasing (or

decreasing) for all $n \geq 0$. In other words, $p_{n+1} > p_n$ ($p_{n+1} < p_n$) for all $n \geq 0$. To verify the monotonicity, the value of p^* as $p^* = 1 \quad (1+m)\xi$

From Eq 2.2 it is found that

$$p_{n+1} - p_n = \frac{p_n(1-p_n)(p_n - p^*)}{(1+m)p_n\xi + (1-p_n)^2}. \text{ (Eq 2.4)}$$

Now, if $p^* = 1 \quad (1+m)\xi \leq 0$, i.e., $\xi \geq 1/(1+m)$, then $p_{n+1} > p_n$ for all $n \geq 0$. If $p^* = 1 \quad (1+m)\xi > 0$, i.e., $\xi < 1/(1+m)$, then $p_{n+1} > p_n$ for all $n \geq 0$ if and only if $p_0 > p^*$. In this case, $p = p^*$ is the unique unstable equilibrium within the interval $(0,1)$. The predicted generations are shown in **Table 2.7**.

Results

Blind tests

The results of the blind tests (**Table 2.2** & **Table 2.3**) show that the sampling 20 samples over the course of 3 days produced a more accurate result. The results of the third blind test show that the PCR system does result in an accurate display of both positive (*Wolbachia* infected) and negative (*Wolbachia* uninfected) results. The sampling size was increased from ten samples to twenty and the sampling style altered to allow for multiple sampling sessions based upon these results. Additionally, the sampling style was changed so that s many females as possible were collected, for each sampling set, and then sub-sampled. The extra females from the set were released back into their cages to be collected and sub-sampled again for the next set (**Table 2.3**). The test of the validity of the PCR system results shows that the system is accurate (**Table 2.4**).

Table 2.2: The results of the first blind bias assessment. The first column denotes the cage number. The second and third columns represent the infection rate estimate achieved by taking 10 and 25 samples, respectively. The final column is the actual infection percentage for each of the three cages.

	Result (10 samples)	Result (25 samples)	Actual
1	40%	28%	50%
2	70%	40%	30%
3	30%	36%	20%

Table 2.3: The results of the second blind bias assessment. This blind test involves taking 25 samples of female ovaries over the course of 3 days. Ten samples were taken the first day and five samples were taken on subsequent days.

	Result	Actual
1	28%	37.5%
2	28%	28.6%
3	16%	15.3%

Table 2.4: The results of the PCR assessment blind test. This blind was run by dissecting all of the live females in each cage, extracting the DNA, and running PCR. Cages one and three had 15/15 samples dissected and cage two had 14/15 samples dissected (one female died before dissection)

Cage	Result	Actual
1	100%	100%
2	71.4%	66.7%
3	33.3%	33.3%

Population Replacement 1

Two strains of a single species, *Ae. aegypti*, were used for this experiment. No less than ten female mosquitoes were randomly selected each generation and tested using PCR targeting the WSP gene. Of the five experimental sets, the 1:4 (Waco male: WB1 male) cage reached full population replacement the quickest at two generations (**Table 2.6**). The percentage of infection in the other three cages (1:0, 1:1, and 1:2) progressed at a relatively stable rate, culminating in full population replacement during various generations (**Table 2.6**). The hatch rates of each of the generations were counted and calculated. At each generation at least 1000 eggs were counted

and the number that hatched was divided by the total number of mosquito eggs to obtain an approximate hatch rate (**Table 2.5**).

Table 2.5*:* The percentages represent the number of eggs for each set of mosquitoes that hatched at each generation.

Generation	Negative Control	1:0	1:1	1:2	1:4	WB1
1	84%	64%	50%	44%	43%	79%
2	82%	56%	53%	34%	74%	60%
3	68%	48%	65%	62%	61%	62%
4	68%	47%	26%	19%	18%	28%
5	80%	47%	53%	57%	56%	58%
6	60%	50%	47%	52%	54%	63%
7	53%	46%	46%	52%	55%	48%
8	51%	43%	58%	62%	63%	44%

Table 2.6: The percentages represent the percentage of each sample set that were found to be infected. The results were obtained using PCR to image a *Wolbachia* surface protein (WSP).

Generation	Negative Control	1: 0	1:1	1:2	1:4
1	0%	50%	70%	80%	100%
2	0%	50%	30%	70%	100%
3	0%	80%	90%	90%	100%
4	0%	100%	100%	100%	100%
5	0%	100%	100%	90%	100%
6	0%	95%	100%	80%	95%
7	0%	100%	100%	100%	95%
8	0%	100%	100%	96%	100%

Mathematical modeling

Based upon the results obtained through use of the mathematical model and a comparison of them to the actual results obtained via completion of the experiment (**Table 2.7**) it is possible to say that the mathematical model is accurate. There is a maximum of a three generation difference between the actual and the predicted generation, with a difference of two generations or less being the norm.

Table 2.7: Table of predicted replacement generations, based on the mathematical model, and the actual generations during which replacement took place.

Waco♂ : WB1 ♂	Predicted Replacement Generation (mathematical)	Actual Replacement Generation (experimental)
1:0	F7	F7
1:1	F5	F4
1:2	F4	F7
1:4	F3	F2
Negative Control	N/A	N/A

Discussion

Blind Tests

The necessity of an increased sample size from 10 to 20 samples is shown through the use of a chi-squared (observed v. expected) statistical analysis. The chi-square observed vs. expected analysis showed that the actual and observed infection frequency was significantly different for the first set of blind assays (**Table 2.2**) that was used to assess the validity of a sampling size of ten (χ^2(df=2)=6.033 p=.049) (α = .05 used for all statistical tests). The result of the chi-squared analysis shows that there is a significant difference between the observed and expected values when only assaying 10 samples that are all taken the same day. By analyzing a further set of 15 samples it was possible to show that taking twenty samples on the same day also displayed a significant difference between the observed and expected values (χ^2(df=2)=6.453 p=.0397). The second sampling size assessment shows that the process of sampling multiple sets,

10 on the first day and five on the subsequent days, is an adequate method for accurately assessing an approximate infection frequency (χ^2(df=2)=0.6173 p=.7344). This supports the use of the second sampling method over the first method.

After assessing that the final sampling method of collecting as many mosquitoes as possible from the cage and sub sampling from that group results in a there being no significant difference between the observed and expected values, an equivalence test was run to ensure that the results of the blind tests were not statistically the same. The epsilon value (calculated by taking 20% of the variance of each group) is considered to be equal for each group, due to all values being very similar. Epsilon is defined as .042. The null hypothesis is that the difference between the groups is greater than the epsilon value, meaning that the groups are not the same. All groups were found to be statistically different from each other (**Table 2.8**).

Table 2.8: The equivalence test for the comparison of each group of samples. For the purpose of this table, each cage has been assigned a different number (1-9). Numbers 1, 4, and 7 designate the groups with a sample size of 10. Numbers 2, 5, and 8 designate the groups where an additional 15 samples were taken after the sample size of 10. Numbers 3, 6, and 9 designate the final group where 25 samples were taken over the course of several days and as many females as possible were collected and sub-sampled for each set (extra females were released back into their cages to be sampled again the next day).

Group X	Group Y	Infection Percentage for group X	Infection Percentage for group Y	Value (difference between means)	Confidence Interval (for value)	P	Null reject (Yes/No)

Table 2.8 cont'd

1	2	50	50	.12	[-0.1806674, 0.4206674]	.6682519	No
1	3	50	37.5	.12	[-0.1806674, 0.4206674]	0.6682519	No
4	5	30	30	0.3	[-0.01372013, 0.61372013]	0.91335	No
4	6	30	28.6	.42	[0.1254568, 0.7145432]	0.9814239	No
7	8	20	20	-.06	[-0.3690375 , 0.2490375]	0.5389628	No
7	9	20	15.3	.14	[-0.1175705, 0.3975705]	0.7379567	No
2	3	50	37.5	0	[-0.2173931, 0.2173931]	0.3736589	No
5	6	30	28.6	.12	[-0.1075098, 0.3475098]	0.7160195	No
8	9	20	15.3	0.2	[-0.006782086, 0.406782086]	0.8969214	No

Population Replacement 1

Based upon this experiment we conclude that the release of additional males that are infected with a strain of *Wolbachia* bacteria can increase the rate at which a population of

uninfected *Ae. aegypti* is replaced by a population of infected individuals. This is shown by the release of WB1 males into cages that contained only a low base number of infected female mosquitoes and an equal number of male and female uninfected mosquitoes. The cage that contained a ratio of 1:4 (uninfected males : infected males) became fully infected at a higher rate than the cage with no additional release of *Wolbachia* infected male mosquitoes(**Table 2.6**).

Mathematical modeling

The mathematical model proved to be relatively accurate. It predicted the replacement generation number, but it needs further refinement in order to predict an accurate infection rate at each other generation. With future refinements the mathematical model designed for this project could be possibly be used for field studies.

The issue with this sample set comes from the fact that there were a minimum of ten females sampled of the 50 collected. The fifty were already a randomly selected subset from the total number of hatched mosquitoes for each cage at each generation. The number of mosquitoes selected for the first subset of 50 may be less than 10% of the total hatched population. However, the sample set of ten was a good starting point, by beginning there, a minimum number of samples to begin the assessment of population replacement rate was established. The issue of the selection size may have been one of the causes in the discrepancy between the number of generations that population replacement with no additional males took place (four to five generations) and previous studies where it took seven generations (Xi et al. 2005b).

CHAPTER 3

Reducing the initial female release frequency threshold for population replacement

Introduction

As mentioned in chapters 1 and 2, Dengue is a very prevalent mosquito-borne disease. Due to its vector it has both a high incidence of initial infection and a risk of re-infection. Over the years, many attempts have been made towards controlling this disease and its mosquito vectors (*Ae. aegypti* and *Ae. albopictus*) and have been met with varied levels of success. Many of the currently used mosquito control techniques have an assortment of objections and difficulties attached to them. These problems range from ethical dilemmas concerning GMOs (Resnik 2012) to the development of resistance to insecticides (Lenormand et al. 1999; Chareonviriyahpap et al. 1999; Rose 2001).

With the objections, hindrances, and dilemmas a different type of control program is needed. That is not to say that the new program would be without its own share of any or all of the afore mentioned types of stumbling blocks, but that each group of troubles would be easier to manage or of a reduced negative impact. One such program is the application of inundative release techniques using *Wolbachia* to replace a Dengue vulnerable population with a disease refractory population (as in chapter 2). While *Wolbachia*-mediated disease control techniques may be difficult to classify under current regulations, as in the case of the release of *Wolbachia* infected mosquitoes in Australia (DeBarro et al. 2011), the lack of genetic modification avoids the ethics struggle associated GMOs and the lack of actual chemicals dodges the potential issues of both insecticide resistance and toxicity brought on by the use of pesticides. In addition, the mosquito release group, while containing some infected female mosquitoes at the first generation, would be entirely made up of males after the initial release. This may reduce

concerns, as the males are not the transmitters of disease. All of these factors combined make the use of *Wolbachia*-mediated disease control rather attractive and the inundative release of male mosquitoes, as opposed to females, can potentially make it more so.

The goal of this experiment was to decrease the threshold number of *Wolbachia* infected female mosquitoes that must be released into a population to achieve fixation of the infection within the population. The hypothesis is that adding male release to the population replacement strategy is a viable way to decrease the number of females required for the infection to reach fixation within the population. This strategy requires the inundative release of *Wolbachia* infected male mosquitoes at each generation.

Materials and Methods

The general procedure is similar to what has been described in the chapter 2 except the below:

Population Replacement design

This experiment entails the use of an extra number of *w*AlbB infected males being released into the population in order to reduce the necessary number of infected females. The idea behind this is that the extra infected males would cause more incidences of CI by mating with the uninfected females. This would reduce the number of uninfected offspring. The infected females are able to mate with both infected and uninfected males successfully, increasing the chance of infected offspring being produced. Depending on the number of infected males released, this would cause a rapid decrease in the uninfected population and, by comparison, an increase in the early generation infection frequency, allowing the infection too persist more readily within a population.

Table 3.1: The experimental design of Population Replacement 2: Reducing the threshold number of *Wolbachia*-infected females necessary for the bacterium to achieve fixation within the population. This table describes the numbers of infected and uninfected mosquitoes released into each of the experimental cages.

WB1 ♀ release %	No. of Waco ♀	No. of Waco ♂	No. of WB1 ♀	No. of WB1 ♂
20%	50	50	13	100
10%	50	50	6	100
5%	50	50	3	100
2%	50	50	1	100
Negative control	50	50	0	0
Total	250	250	23	400

There were a total five cages used in this experiment. The cages were set up with varying numbers of *Wolbachia* infected females, with the exception of negative control (**Table** 3.1). This experiment used a system of single *Wolbachia* infected female release in generation zero (F0) and continued inundative release of infected males. For each generation, once the eggs were collected ten adult females were selected at random, dissected, and their ovaries were collected for use in PCR.

Mathematical modeling

The equation for predicting the generations at which the replacement would take place, depending on the size of the initial female seeding population, is a modification of the equation

used for the prediction of the generations of population replacement in increasing the rate of population replacement. These generations were predicted using the equation that follows: $a_n = 50(1-p_n)$, $A_n = 50p_n + 100$, $b_n = 50(1-p_n)$, $B_n = 50p_n$. When n>0. (Eq 3.1)

Where a_n is the number of *uninfected males,* A_n is the *infected males,* b_n is the *uninfected females,* and B_n *infected females.* The number of uninfected females in the next generation G_{n+1} corresponds to the matching frequency between the number of uninfected male and female mosquitoes, and therefore corresponds to $a_n b_n = [50(1-p_n)]^2$. The number of infected females also corresponds to the matching frequency of infected male and female mosquitoes and then is multiplied by the infection cost, $\xi(a_n + A_n) \times B_n = 7500 p_n \xi$. Together, these numbers define the total infection frequency for G_{n+1}, by calculating:

$$p_{n+1} = \frac{3p_n\xi}{3p_n\xi + (1 \quad p_n)^2} \text{ for all generations n > 0. (Eq 3.2)}$$

To calculate the infection frequency for generation 0 to generation 1, it is necessary insert the actual number of males and females into each of the equations: $a_n = 50(1-p_n)$, $A_n = 50p_n + 100$, $b_n = 50(1-p_n)$, $B_n = 50p_n$, meaning $a_n = 50$, $A_n = 100$, $b_n = 50$, and $B_n = f$. This results in the equation:

$$p_1 = \frac{3f\xi}{3f\xi + 50} \text{ (Eq 3.3)}$$

For each given fitness cost ξ and seeding number of infected females f the infection frequency p_{n+1} is determined by the infection frequency p_n in the previous generation. This equation follows the assumptions that 1) there is an equal chance that any female will mate with

any male, she will not discriminate based on infection presence or absence, 2) there is equal sex determination for each experimental generation, the probability that any one egg will be male or female is .5, 3) there is complete CI sterility, all incompatible matings will result in no offspring, and 4) 100% maternal transmission, all offspring of infected females will have the same infection type as the female. This model is designed to take into account a varying number of infected females being released into the population at only generation 0 and the fixed number of infected males that are released at every generation.

The same concepts of uniqueness and monotonicity that were applied to the equation for population replacement 1 were applied to equation 3.2, resulting in

$$p^* = 1 - 3\xi .$$

Then from (3.2) we find

$$p_{n+1} \quad p_n = \frac{p_n(1-p_n)(p_n - p^*)}{3p_n\xi + (1-p_n)^2}.$$

Now, if $p^* = 1 - 3\xi \le 0$, i.e., $\xi \ge 1/3$, then $p_{n+1} > p_n$ for all $n \ge 0$. If $p^* = 1 - 3\xi > 0$, i.e., $\xi < 1/3$, then $p_{n+1} > p_n$ for all $n \ge 0$ if and only if $p_0 > p^*$. In this case, $p = p^*$ is the unique unstable equilibrium within the interval $(0,1)$.

Results

Based on mathematical modeling it is possible to show that the introduction of *Wolbachia* infected males will lower the threshold number of infected females necessary for population replacement (**Table** 3.2). The model shows that a female release that is as low as 2% of the total female population will still result in complete population replacement when combined with continued inundative *Wolbachia* infected male release.

The use of inundative infected male release as a method to lower the threshold number of necessary infected females needed to achieve population replacement is demonstrated using the *Anopheles stephensi* mosquito (**Table** 3.3) (Bian et al. 2013)

Table 3.2: The expected generations when the infection frequency has reached 99.9%. The predicted generations were calculated using the mathematical model (Eq. 3.1 through 3.3)

Female infection Frequency	Predicted generation
20%	F4
10%	F5
5%	F6
2%	F7
Negative Control	No population replacement

Table 3.3: Predicted and actual replacement generations achieved using inundative release of *Wolbachia*-infected male *Anopheles stephensi* mosquitoes to reduce the number of infected female mosquitoes necessary for complete population replacement.

Infected percentage of ♀	Mathematically predicted replacement generation	Experimentally achieved replacement generation
5%	F7	G8
10%	F6	G8
20%	F5	G7

Discussion

Infected male inundative release can potentially decrease the number of infected females necessary for a seeding population. At present, a minimum of a 20% *Wolbachia* infected female, calculated using the number of infected females divided by the total number of females in the entire population, release is necessary for full population replacement. The equation designed for this projected indicates that the number of *Wolbachia* infected females can be reduced by inundative release of infected males at every generation. This reduction in the number of required females results in a more socially acceptable program. The decreased number of infected females being released does bring with it the potential for a decreased rate at which population replacement will take place. Applying the same concept used in Population Replacement 1, it may be possible to limit the rate decrease by altering the number of infected males that are released into the population.

In support of the utility of inundative male release and the mathematical model the comparison between the generation of replacement generated through mathematical modeling and the generation of replacement that was experimentally derived has been provided (**Table** 3.3). This table shows that the model is relatively accurate, but still might need some fine tuning. This may be due to a need to further assess fitness costs and population dynamics associated with the novel infection of *An. stephensi* with *w*AlbB. So, while not 100% accurate when comparing predicted and actual replacement generations, the model still supports both the ability of this inundative release technique to reduce the necessary number of females and the applicability of the model to different genii of mosquito. It is likely that the model will need to be fine tuned for each new genus of mosquito it is applied to. The usefulness of the model for more than one genus of mosquito can makes it a very practical research tool in both laboratory and field studies.

In the lab it could be used to assess viability of and created a plan for a replacement strategy. In field studies it could be used to help predict generations of replacement, optimize the replacement rate, and help decide an approximate number of mosquitoes to begin the replacement with.

CHAPTER 4

Characterization of Cytoplasmic incompatibility Phenotypes with Triple infected *Aedes albopictus*

Introduction

Aedes albopictus (Skuse) is the secondary vector of dengue virus. It is a mosquito that has adapted to the urban environment provided by human habitation, as well as the sub-urban and semi-rural areas (Knudsen 1995). Due to the lack of vaccines for dengue and the spread of its vectors, it is imperative to come up with effective disease control strategies. Many methods have been explored for the control of disease vectoring species like *Ae. albopictus*. These control techniques focus on both larval and adult stages of the mosquitoes, such as larvicides and adult insecticides, sterile male release techniques, biological control agents, and genetically modified mosquito release (Paupy et al 2009). One strategy with potential is the use of *Wolbachia* to control the spread of dengue. In studies with *Aedes aegypti* it has been shown that *Wolbachia* infection inhibits the transmission of dengue (Bian et al. 2010) and that it takes seven generations in *Ae. aegypti* to drive an infection into the population when a specific minimum initial infection threshold is met (Xi et al. 2005b). There are hopes of a similar response in *Aedes albopictus*.

Ae. albopictus naturally carries two types of *Wolbachia*, wAlbA and wAlbB (Kittayapong et al. 2002) and studies have shown that it is possible to transfer infection types within *Ae. albopictus* via microinjection of the embryo (Xi et al 2005a). There have been different types of *Wolbachia* used to generate a triple infected line (Fu et al. 2010). However, the focus thus far has been in using single infected males that contain an incompatible infection type for population suppression (Calvitti et al. 2010; Laven 1967).

Ae. albopictus exhibits a unidirectional type of CI called additive CI (**Figure** 1.3). Additive CI occurs when there are at least two *Wolbachia* infection types within individuals of the population. This type of crossing displays an increasing percentage of total crossings that result in CI directly related to the increasing number of infection types. Due to being a type of unidirectional CI, the mod/resc system holds true. The *Wolbachia* modify the sperm and they make it so that an egg with the same infection type can rescue it (Werren 1997), as discussed in chapter 1.

Despite the evidence for the ability of *Wolbachia* to inhibit dengue, it is still a secondary disease vector for dengue virus. There is hope that a new, third, type of *Wolbachia* infection may prevent the transmission of dengue by *Ae. albopictus*. In order to effectively design an experiment to drive this bacterium into the population and assure that there are three infection types within the population, phenotypic characterization of the CI must occur.

Materials and Methods

The Houston line of *Ae. albopictus* mosquitoes was used for this experiment. Through microinjection techniques a line (HC) of *Ae. albopictus* mosquitoes was developed that contained three types of *Wolbachia*: *w*AlbA and wAlbB (from *Ae. albopictus*) and *w*Pip (from *Culex pipiens (*Hertig 1936). It was generated via infected cytoplasm microinjections to the embryo of the *Ae. albopictus*, using cytoplasm from the *Culex pipiens*, similar to how the WB1 line was created (Xi et al 2005b).

Once the lines were established and enough eggs were generated to maintain the lines, crossing experiments commenced. This type of crossing experiment used three lines of *Ae. albopictus* mosquitoes, Houston Tetracycline treated (HT), Houston wild type (Hou), and

Houston triple infected (HC). The HT line was the uninfected line. It was generated by feeding Hou mosquitoes tetracycline to wipe out the natural *Wolbachia* infection types (Dobson and Rattanadechakul 2001). The HC line was created via microinjection and the experiment took place during the eighth generation of the HC line. The Hou line is the wild type and originated in Houston, Texas (1986).

The cages used for this study were medium sized Tupperware bowls modified so that there was a hole in the side of the bowl and a tube of cloth netting was attached to cover the hole and allow the cage to seal. The top was cut so that only the rim of the lid remained and was used to hold plastic mesh netting in place to allow for blood feeding and sucrose feeding.

All mosquito eggs were stored at a constant temperature and moisture before hatching (average 26.9C) and moisture (70% relative humidity). The eggs were stored for no more than three months because storage for a longer amount of time may result in a sharp decline in the hatch rate. The eggs were laid on egg paper over the course of two to three days. The egg papers are pieces of water absorbent brown paper that was cut to fit the egg cups from a 9x11” sheet (University Stores, Michigan State University, East Lansing, MI, USA). Four egg papers were placed into a 5 ½ oz plastic soufflé cup (Solo Cup co, Lake Forest, IL, USA) and then moistened with water.

The eggs were hatched in a 6% liver powder (Now Foods, Bloomingdale, IL, USA) and water mixture that had been fermented for at least one week to create deoxygenated water and then was diluted to between 30% and 50% with purified water. The water mixture and the egg papers were left in the water overnight in a plastic hatch tray (8.2”x8.34”x2”, 49oz, Pactiv Foodservice/Food Packaging, Lake Forest, IL, USA) and the emerging larvae were split into new hatch trays into groups of approximately 100 larvae per tray. Fresh water was added to dilute the

deoxygenated water further. The larvae were fed a 6% liver powder water mixture each day. After 5-6 days the larvae pupated and were collected from the pans for use in the experiment.

The pupae were placed into individual test tubes, by sex, to provide enough virgin males and virgin females for each cage. The pupae were sexed via size comparison. The males tend to be noticeably smaller than the females. The mosquitoes were checked for sex after emergence and before release in the cages. This was a simple matter of comparing antennae and proboscis structures. After emergence, the males and females were released into the cages in preset crosses. Each cage received ten males and ten females. The crossing types and expected pattern of CI are shown in **Table** 4.1.

Table 4.1: Crossing patterns and expected CI responses for all possible CI crossings of HT1, Hou, and HC *Ae. albopictus* mosquitoes.

Male \ Female	HT	Hou	HC
HT	Compatible	Compatible	Compatible
Hou	CI	Compatible	Compatible
HC	CI	CI	Compatible

After release into the cages, they were allowed to mate until they reached 7-10 d post emergence, and they were subsequently blood-fed on live mice. After the blood meal the mosquitoes were given two days to rest for ovigenesis, and one 5 ½ oz cup was placed into each cage to collect eggs. Approximately 2 days after the egg cups were placed in the cages they were removed and placed inside of a hatch tray under a damp paper towel for 2 days. The eggs were then dried for around 7 days and then hatched. After 2 days the egg papers were pulled out of the water and the hatch rate was counted. The blood feeding was performed a second time for the same cages and

egg collection process was repeated. The second set of eggs was then hatched and the hatch rate was counted (**Figure** 4.1).

Results

The crossing results were as expected as shown by the comparison of the table (**Table** 4.1) and figure (**Figure** 4.1). The results show that *w*Pip is incompatible with a double infection of *w*AlbA and *w*AlbB. The triple infected line induced unidirectional CI and the *w*Pip infection is incompatible with a double infection of *w*AlbA and *w*AlbB. The expected incompatible cross types resulted in a hatch rate of zero. There were some lower hatch rates associated (**Figure** 4.1) with the Hou and HC lines, but these rates were not significantly different when compared to the hatch rates of the other crosses (**Table** 4.2 and **Figure** 4.2).

Figure 4.1: The average hatch rates for all crosses. The *# represents how many replicates of each cross were run. The HC line was in its eighth generation during this experiment.

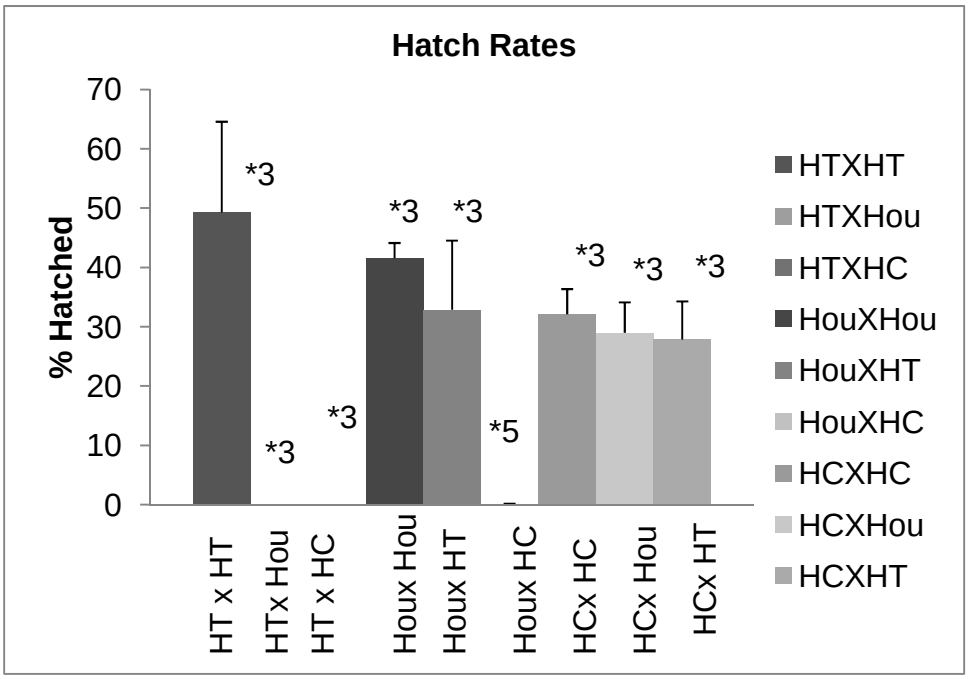

Figure 4.2 The one way analysis of the asin proportion of hatchings by crossing showing that none of the hatchings are significantly different from each other.

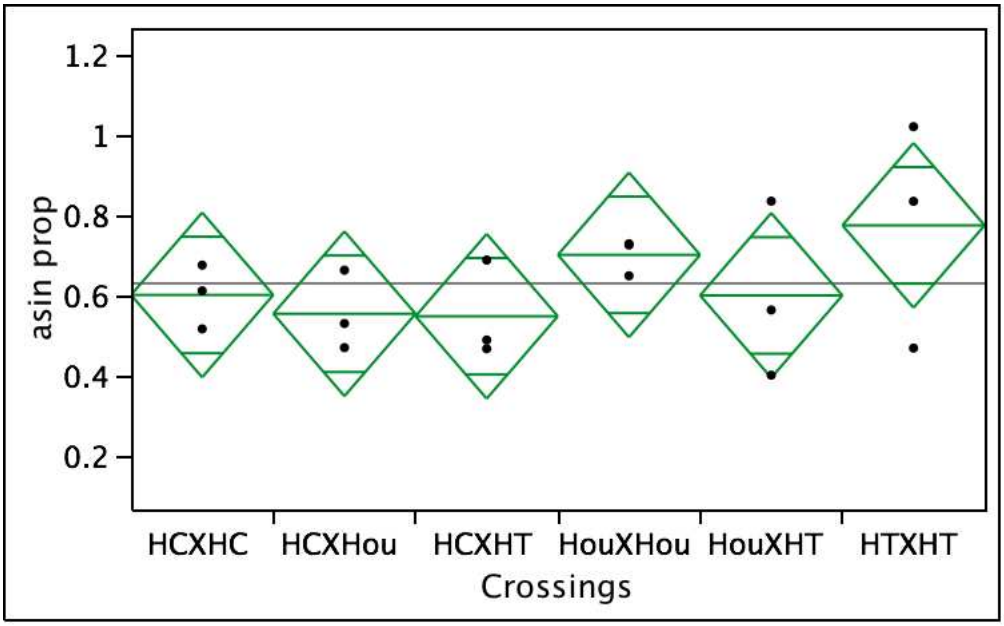

Table 4.2: The analysis of variance for the *Ae. albopictus* CI crossing hatch data. This test shows that there is no significant difference between the average hatch rates produced by each of the CI crosses.

Source	DF	Sum of Squares	Mean Square	F Ratio	Prob > F
Crossings	5	0.12012592	0.024025	0.9045	0.5093
Error	12	0.31875256	0.026563		
C. Total	17	0.43887848			

Discussion

It is important to note that this third type of infection within the *Ae. albopictus* system using *w*Pip is novel. There has been at least one other triple infection type used for *Ae. albopictus* mosquitoes, but the type of *Wolbachia* was *w*Ri from *Drosophila simulans* (Fu et al 2010). This infection characterization showed the possibility of using triple infected *Ae. albopictus* to act as the CI mechanism for a population replacement experiment. There have been other studies involving the introduction of *w*Pip into a population of *Ae. albopictus*, however these studies introduced the infection into a *Wolbachia* negative line of the mosquito to create a single infected line of the species (Calvitti et al. 2010). That study assessed the maternal inheritance of the infection, the fitness effects, and the CI expression within the new transinfected line. They found that there was a significant fecundity cost for the new single infected line; however continued selection may reduce the fitness costs. There was complete CI sterility, and 100% maternal transmission (Calvitti et al 2010).

It is also important to note that there are no known systems that contain four types of *Wolbachia* within the same individual. This may be due to a combination of stress upon the host caused by supporting multiple infection types or that not all *Wolbachia* types have an additive effect and those that do not often compete with each other within the host and one or more infections are lost.

There is great disease control potential for this triple *Wolbachia* infected line based on the results of this experiment. It has been shown that the *w*Pip infection type is resistant to dengue virus in both the midgut and salivary gland (Xi et al. unpublished data). Due to this resistance it is possible that this third infection type within the *Ae. albopictus* system could be used to clean the mosquitoes of the dengue virus and halt the disease transmission via the secondary vector.

The selection of an infection type that matches well with desired characteristics for using a *Wolbachia* driven system is extremely important. In order to make this type of selection, the infection and the fitness costs, maternal transmission rate, CI sterility rate, and the additive effects within the population must be observed. All of these factors can influence the effectiveness of both population replacement and population suppression experiments. If the fitness costs are too high, the maternal transmission to low, or if the CI sterility is incomplete it can reduce the effectiveness of the vector control strategy and, could cause the project to fail.

CHAPTER 5

Mosquitoes, *Wolbachia*, and cytoplasmic incompatibility: Conclusions and future directions

Conclusions

Wolbachia has been proven to inhibit the transmission of dengue virus in mosquitoes (Lu et al 2012; Walker et al 2011; Hoffmann et al 2011; Iturbe-Ormaetxe et al 2011; Blagrove et al. 2011). Thus, it is important to find an effective way to drive this bacterium into disease vector populations. We observed that the release of additional infected males into the population can accelerate the population replacement. These additional males cause an increase in the frequency of incompatible matings and result in the decline of the uninfected population. There is a direct correlation between an increased ratio of infected males and an increased rate at which population replacement will occur, as shown by equation 1. Therefore, it is possible to conclude that an increased number of *Wolbachia* infected males released into a population will cause an increased rate of population replacement. In addition, we have made mathematical modeling to predict in which generation population replacement will occur with this additional male release strategy.

We also found that *w*Pip is incompatible with *w*AlbA and *w*AlbB infection types. Due to *w*Pip being incompatible with *w*AlbA and *w*AlbB, the triple infected *Ae. albopictus* line generated for the studies within the Xi lab causes unidirectional CI when crossed with the super-infected wild type mosquito and the tetracycline treated, *Wolbachia*-negative, *Ae. albopictus* line. Together with PCR diagnosis result, the CI cross data confirm that the HC triple infected *Ae. albopictus* line does actually contain three types of *Wolbachia*.

Overall, both population replacements in *Ae. aegypti* and characterization of CI hatch phenotypes in triple infected *Ae. albopictus* are important steps toward disease control. Both of these vectors are now wide spread invasive species in the majority of tropical and subtropical areas around the world. There are still many research directions that need to be explored to further this style of disease control.

Future Directions

The next step for the first population replacement experiment is to repeat the experiment to gather more data and ascertain the most efficient ratio of male release to achieve population replacement and to increase the experiment size to get a better view of a larger population, such as a field population. In laboratory cage tests are often designed to assess the feasibility of a study within a controlled environment and they are designed to be used a model to base field-cage and field studies upon. Once a field-cage study, is completed and the results are confirmed the data can be used for projects like increasing the predictive power of the mathematical model, modification of the model to account for a different species, or population replacements of other species of vector containing *Wolbachia*,.

These results can be used to build several models that can be applied to other disease vectors that either do not naturally carry *Wolbachia* or that do not carry the particular type of *Wolbachia* being introduced into the population. Considering that some types of the bacteria can shorten the life span of the vector in question, as well as be used to drive desirable traits into the population (Werren et al. 2008), it is a small leap to extend this idea to other types of disease vector. Research delving into the possibility of controlling diseases that have a specific incubation period within the insect before they are able to be transmitted has begun. This research relies on the alteration of the age population structure by shortening the life-span of the insect with

Wolbachia (Cook et al. 2008). The data collected in this experiment may make it easier for such studies to predict how many *Wolbachia* infected insects must be released to complete the replacement and determine ways to reduce the number of blood-meal requiring females that must be released.

There are several future directions associated with the triple infected *Ae. albopictus* line. The first project should be fitness costs studies, such as fecundity, survivorship, etc similar to what was done by Calvitti et al. (2010) for the single *w*Pip infected *Ae. albopictus* line and studies that look at the stability of the three infection types over time. Once these studies have been completed it will be possible to set up several cage population replacement experiments. Due to *w*Pip being resistant to dengue virus (Xi et al. unpublished) it is a good candidate for future field replacement studies. Another future project to be completed, after the cage studies, is a field population replacement, such as what was done using *w*Mel infected *Ae. aegypti* in Australia, *w*MelPop was also investigated, but the fitness costs for infected mosquitoes were too high and the infection had trouble spreading in the population (Hoffmann et al. 2011).

The single infected or the triple infected lines of *Ae. albopictus* can be used for population suppression similar to the suppression of *Culex pipiens fatigans* (Laven 1967), so long as the infection type makes the released mosquitoes incompatible with the current population. The suppression strategies (Dobson et al. 2002) can be used in tandem with population replacement to create a more effective control strategy or the suppression strategies can be used to create an artificial unstable equilibrium that continually drives the population size down over several generations (Dobson et al. 2002).

Population replacement studies are applicable in other species besides *Ae. aegypti*. There have already been studies in population replacement using *Anopheles stephensi*. A line of this

species has been artificially infected with *Wolbachia w*AlbB and population replacement studies have been worked on to assess the lowest number of infected females that can be released into the population and have the infection reach fixation (Bian et al. 2013). This work was undertaken due to the ability of *w*AlbB to cause the *An. stephensi* mosquito to be resistant to *Plasmodium falciparum* (Hughes et al. 2011).

Another option that is currently undergoing study is the use of transgenic mosquitoes. In various studies there have been modifications to the genome of certain mosquito vectors. These modifications were designed to elucidate the purpose of a variety of genes within the mosquito system and find possible methods for the control of the spread of disease. An example of one of these types of studies involves an alteration of the REL1, which is related to the Toll immune pathway of insects. The over expression of which causes an up-regulation of Spatzle and immune response (Bian et al 2005), meaning an up-regulation of the Toll pathway. The Toll pathway plays a significant role in the regulation of the *Ae. aegypti* mosquito's resistance to dengue infection and its ability to limit the viral infection (Xi et al. 2008). Should the fitness costs associated with transgenic mosquitoes, not just those that were modified using the REL1 gene, (Irvin et al 2003; Catteruccia et al. 2003; Moreira 2003) be mitigated or alternate genes with a similar effect but lacking the fitness cost be discovered this could easily become a valued disease control strategy. The other issues with this strategy involve mitigating the biosafety risk, regulation approval for field releases, public approval, and environmental hazards (Sperança and Capurro 2007).

Due to the prevalence of diseases like dengue, it is important to find an effective control strategy. Every year thousands died from dengue due to the lack of vaccines and treatments to combat this disease. Vector control is the only real option for controlling the spread of dengue

and dengue hemorrhagic fever. The development of resistance to various pesticides in mosquitoes and the lack of a self-sustaining solution make the *Wolbachia*-mediated disease control strategy very promising. *Wolbachia*-based population replacement is unique in that it is naturally occurring, self-sustaining, and host specific, thus it will not affect non-target species. All of the above make the use of *Wolbachia* for disease control a very attractive alternative and future studies, as described above, are expected to further improve the efficiency and efficacy of *Wolbachia*-based population replacement strategies.

APPENDIX

APPENDIX

RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed below have been deposited in the named museum as samples of those species or other taxa, which were used in this research. Voucher recognition labels bearing the voucher number have been attached or included in fluid preserved specimens.

Voucher Number: ___2013-06_____

Author and Title of thesis:

Wolbachia-Mediated Population Replacement in Dengue Mosquito Vectors By Shawna Ryan

Museum(s) where deposited:
Albert J. Cook Arthropod Research Collection, Michigan State University (MSU)

Specimens:

Family	Genus-Species	Life Stage	Quantity	Preservation
Culicidae	*Aedes aegypti*	adult	10	pinned
Culicidae	*Aedes aegypti*	adult	10	alcohol
Culicidae	*Aedes albopictus*	adult	10	pinned
Culicidae	*Aedes albopictus*	adult	10	alcohol

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