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EFFECTS OF METHOPRENE ON THE SURVIVORSHIP  
AND FECUNDITY OF ADULT AEADES MOSQUITOES: A STRATEGY FOR  
INACTIVATING RELEASED MOSQUITOES

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THESIS

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A thesis submitted in partial fulfillment of the  
requirements for the degree of Master of Science in the  
College of Agriculture  
at the University of Kentucky

By

Peter Joseph Brabant III

Lexington, Kentucky

2012

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

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## ABSTRACT OF THESIS

### EFFECTS OF METHOPRENE ON THE SURVIVORSHIP AND FECUNDITY OF ADULT AEADES MOSQUITOES: A STRATEGY FOR INACTIVATING RELEASED MOSQUITOES

Methoprene is a Juvenile Hormone (JH) analogue commonly used for the control of mosquito larvae. When applied to a mosquito breeding site, methoprene enters the haemolymph, where it mimics the function of JH and interferes with normal metamorphosis, resulting in larval mortality. Methoprene is commonly used for the control of larvae and has not been used as an adulticide, due to an absence of acute effects. This study evaluated possible chronic effects caused by the exposure of adult *Aedes* mosquitoes to methoprene. Methoprene was applied, in both technical grade and the commercially available Altosid®, topically to adults through droplet application on the abdomen and as a spray application. Mosquitoes were examined for treatment effects on ovary development, adult male and female mortality, and fecundity. The results demonstrate that relatively high doses are required to affect adult survivorship. In contrast, significant impacts on both fecundity and egg hatch were observed for females treated at the lower dosages. I discuss the results in relation to autocidal strategies for mosquito control in which the release of fecund females is to be avoided.

KEYWORDS: *Aedes*, Methoprene, Insect Growth Regulator, Fecundity, Vitellogenesis

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Peter J. Brabant III

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8/24/2012

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AND FECUNDITY OF ADULT Aedes MOSQUITOES: A STRATEGY FOR  
INACTIVATING RELEASED MOSQUITOES

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8/24/2012

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Dedicated to my dearest Rhyannon,  
without whom I would never have succeeded.

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## Chapter One

### Introduction

The *Wolbachia* biopesticide approach uses repeated, inundative releases of male mosquitoes to control populations of mosquitoes. A biopesticide is defined as the application of a microorganism with the purpose of control or suppression of a pest species (Hynes and Boyetchko 2005). Prior examples of this method include the use of *Bacillus thuringiensis israelensis* (Bti) for the control of mosquito larvae, where granules containing the crystalline toxins created by the Bti bacteria are used to treat mosquito breeding sites (Boisvert and Boisvert 2000). The *Wolbachia* biopesticide treatment differs from other biopesticide applications by the use of infected mosquitoes to carry the microorganism to the target mosquito species. Released mosquitoes have been artificially infected in the laboratory with an obligate intracellular bacterial symbiont called *Wolbachia* (Atayame *et al.* 2011). When a male mosquito carrying a novel strain of *Wolbachia* mates with a wild female mosquito that does not carry the same *Wolbachia* infection, the female becomes incapable of producing offspring due to a mechanism called Cytoplasmic Incompatibility (CI) (Brelsfoard *et al.* 2009). CI causes karyogamy failure and arrested embryonic development, preventing the production of offspring (Werren 1997). By increasing the number of incompatible mating, the *Wolbachia* biopesticide method leads to mosquito population suppression (Dobson 2003).

For this method to effectively reduce the numbers of wild mosquitoes, a large number of male mosquitoes must be released. In laboratory trials, this number has been determined to be approximately 15 times the number of mosquitoes in the wild

population (Mains 2012). Release of fertile females is to be avoided, not only because they can transmit disease but also because this can cause population replacement. Fertile *Wolbachia*-infected females would be able to mate successfully with the released males. This gives the accidentally released females a reproductive advantage and ability to displace wild populations (Xi *et al* 2005). In this case, continuing to release males infected with the same *Wolbachia* infection type will no longer have any effect on the mosquito population, due to the presence of the infected females.

To prevent the release of infected females, methods have been created to separate male and female mosquitoes as pupae in the laboratory prior to release. This method is effective, though not perfect. It is possible for female mosquitoes to be missed, and samples must be sorted by hand in order to confirm that no female mosquitoes are mistakenly released (Mains 2012). This process is slow and time inefficient. There is a need for a more rapid and efficient method of assuring that population replacement does not occur.

One potential method of preventing accidental population replacement is to ensure that any females that are released are incapable of producing offspring. One could ensure this by performing a chemical treatment which specifically targets female mosquitoes while not affecting males. This treatment would focus on the physiology of the adult female mosquito and prevent the mosquito from reproducing. For a chemical treatment to be successful, the treatment cannot have a negative effect on male mosquitoes. The *Wolbachia* bio-pesticide approach functions by increasing the numbers of incompatible males in the environment, and therefore relies on the released males surviving long enough to mate.

A potential candidate for this treatment is juvenile hormone (JH). In mosquito physiology, JH is required during the early stages of female reproductive development (Klowden 2002). This hormone controls events during previtellogenic stage t (Raikhel *et al.* 2002). During vitellogenesis, oocytes develop until they reach a resting length of 100  $\mu\text{m}$  (Noriega 2004). At this point, development of oocytes halts, until the mosquito takes a bloodmeal (Raikhel and Dhadialla 1992). Once the mosquito takes a bloodmeal, JH levels become reduced, and the ovary of the mosquito begins to secrete ecdysone (Raikhel 1992). Ecdysone is the primary hormone which directs development of the oocytes in the ovarioles (Hagedorn 1989). In male mosquitoes, JH has been shown to be associated with the male accessory gland (Borovsky *et al.* 1994). The mosquito male accessory gland is contribute proteins to seminal fluids which improves seminal retention in females after mating (Leopold 1976).

Juvenile hormone analogs (JHA) are used already in the field for the control of mosquitoes. Synthetic JHA is applied to the water, for control of larval mosquitoes. This synthetic JHA is a chemical known as methoprene, which has been in use since 1975 (Csondes 2004). While the larval mosquito develops, methoprene interferes with ecdysone induced gene expression, which prevents midgut remodeling in the pupal mosquito and kills the mosquito before it molts into adult (Wu *et al.* 2006). Methoprene has been shown to be a strong larvicide, and kills most larvae that come in contact with it (Norland and DeWitt 1975).

While the effect of methoprene on mosquito larvae is well known, little is known about the effects methoprene would have on adult physiology. The purpose of this study is to quantify the effects of the Insect Growth Regulator (IGR) methoprene on adult

mosquitoes, and investigate the potential of methoprene as a method for selectively inactivating female mosquitoes, while leaving male mosquitoes unaffected.

## **Objectives/ specific aims**

My thesis research is focused on the development of a novel pesticide application using the IGR, methoprene, to lower the fecundity of female *Aedes* mosquitoes. Male and female survivorship was also assessed to determine methoprene effects. The optimum treatment would also have a negligible effect on male mosquitoes.

In respect to inundative releases, the specific aims are:

1. Test whether methoprene application will affect male and female mortality and the fecundity of the model species *Ae. aegypti*
2. Test for a similar effect of this method on *Ae. polynesiensis* and *Ae. albopictus*
3. Test the application of a commercially available form of methoprene, Altosid®, on all three mosquito species on male and female mortality and fecundity



## Chapter Two

### **Methoprene effects on survival and reproductive performance of adult female and male *Aedes aegypti* (Diptera: Culicidae)**

#### **Introduction**

The yellow fever mosquito, *Ae. aegypti* L., is the principal vector, worldwide, of dengue fever. Dengue affects an estimated 50-100 million people per year, resulting in greater than 20,000 deaths annually (Gibbons and Vaughn 2002). Currently no vaccine or specific therapeutic treatment for dengue exists. Novel control methods are thus sorely needed. Current measures against *Ae. aegypti* consist of breeding source reduction and application of chemical insecticides. However, both strategies are complicated by the behavior of this vector and its use of small, dispersed, inaccessible and cryptic bodies of water (Gubler and Clark 1996).

The self-delivering feature of Sterile Insect Techniques (SIT) type approaches make them attractive against container breeding mosquitoes, such as *Ae. aegypti* (Alphey *et al.* 2009). One such approach is based on *Wolbachia*-induced cytoplasmic incompatibility and is being developed for various mosquito species (Atyame *et al.* 2011). The *Wolbachia* biopesticide approach releases male mosquitoes which have been infected with a strain of symbiotic *Wolbachia* bacteria. When these infected mosquitoes mate with wild females, the presence of the bacteria prevents the females from producing offspring. With repeated releases of infected male mosquitoes over time, the wild population could potentially be reduced (Brelsfoard *et al.* 2008).

For applied strategies based on inundative mosquito releases, it is typical to remove females, leaving males only in the release materials. This is important because female mosquitoes bite and transmit disease, but female releases can also represent an experimental complication. For the *Wolbachia* biopesticide approach, the release of many females has the potential to establish the *Wolbachia* infection of the release strain at the release site, which could lead to compatibility with subsequent male releases (Dobson 2003). For additional approaches, such as those based on transgenic mosquitoes, female release may be undesirable from a regulatory standpoint (Benedict and Robinson 2003).

The potential of unintended female release has led to the development of methods for improved accuracy of sorting and removal of females from males (Catteruccia *et al.* 2005). Additional approaches are being developed for the inactivation of females, such that if a female is released, she is short lived and/or unable to reproduce. An example of the latter is the exposure of release material to low doses of radiation, which has no detectible effect on males but sterilizes females (Brelsfoard *et al.* 2009). Here we examine an additional approach, based on the pre-treatment of release material with a chemical that potentially affects females but not males.

Methoprene is a Juvenile Hormone (JH) analog that was first registered as a biological pesticide in 1975 and has proven effective in the control of mosquito larvae (Norland and DeWitt 1975). Methoprene has been approved by the World Health Organization as relatively safe and nontoxic for humans and other vertebrates, including use in drinking water (Garg and Donahue 1989).

JH plays an important role in the regulation of development of insects. Molting between larval instars is initiated by ecdysone. During the molt, the presence of high JH levels prevents metamorphosis to the pupal and adult life stages. With high JH levels, the larva molts into the next larval instar (Klowden 2002). With low JH levels, an increase in ecdysone levels initiates a cascade of events resulting in a molt to the pupal stage (Borovsky and Van Handel 1979). The presence of additional methoprene in the larval mosquito interferes with ecdysone induced gene expression, and prevents midgut remodeling during the pupal stage, causing the death of the larvae (Wu *et al.* 2006).

The effect of methoprene on imagos has received less attention despite known effects of JH on adult physiology. Methoprene, in combination with fipronil, was associated with a significantly lowered fecundity and egg viability in fleas (Franc *et al.* 2007). In mosquitoes, an association has been shown between methoprene and vitellogenesis (Flanagan and Hagedorn 1977). Following eclosion, JH initiates follicular development, until approximately one day after eclosion (Gwadz and Spielman 1973), at which time the follicles reach a “resting stage” of about 100  $\mu\text{m}$  in length. After blood-feeding, JH levels drop, increasing again after the eggs are laid (Noriega 2004). During this period of lowered JH titre, follicles in the resting stage are exposed to ecdysone, which causes them to fully develop (Hagedorn *et al.* 1979). Furthermore, application of JH causes decrease in ecdysteroid levels in adult female mosquitoes following a blood meal (Bai *et al.* 2010). In one prior study, methoprene applied to adult female *Ae. aegypti* resulted in follicular blockage (Judson and Lumen 1976). In the latter study, while an effect on egg laying was described, fecundity was not quantified.

The objective of this study was to analyze the effects of methoprene on the fecundity and mortality of adult *Ae. aegypti*, focusing on the impact of differing methoprene concentrations across two gonotrophic cycles. I tested for an effect of methoprene application on female reproduction and also examined whether application of methoprene to males at the same levels affects male survival.

## **Methods**

### **Methoprene**

Concentrations of 1%, 0.1% and 0.01% methoprene were made by diluting 99.97% technical grade methoprene (Central Life Sciences, Dallas, Texas) with acetone. The control was acetone alone.

### **Mosquitoes**

Mosquitoes used in the droplet and spray methoprene application studies were the Liverpool strain of *Ae. aegypti*, obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) in June 2009. For male competitiveness experiments, the strains used were Waco, which is naturally uninfected with the *Wolbachia* symbiont, and WB1, which has been infected with *Wolbachia* by microinjection (Xi *et al.* 2005). The colonies were maintained as previously described (Gavotte *et al.* 2009). In brief, seed germination paper (Anchor Paper, Saint Paul, Minnesota) which mosquitoes had oviposited on were submerged in 500 ml water and emergent larvae were reared in

optimal conditions with the daily addition of food (6.0% liver powder solution). All mosquitoes used in this study were kept at 27°C and 70% RH, with a 14:10 hour light:dark cycle inside an incubator (Model I-36VL, Percival Scientific, Perry, IA). Cages of adults were provided constantly with a 10% sucrose solution.

### **Droplet Application**

Mosquitoes were aspirated from the maintenance colony 3 d after eclosion using a motorized aspirator, and then anesthetized with chloroform for 30 s. Each mosquito was placed ventral side up using soft forceps, and 1  $\mu$ l of test solution was applied directly to the abdomen using an Eppendorf 2  $\mu$ l micropipette (Hamilton, Reno, NV). Fifty dosed male and female mosquitoes were then moved into each of four cages (one for each experimental concentration, as well as one control cage treated with acetone) constructed of 2.5 liter wax paper buckets (Solo Cup, Lake Forest, IL) with a sleeve attached to the side constructed of medical stockinette (Dynarex, Orangeburg, NY). Mosquitoes were given an hour to recover from the chloroform before cotton soaked in sucrose was provided for sugar feeding.

Three days after dosing, mosquitoes were provided one anesthetized mouse (IACUC #00905A2005) to feed on for 15 min. After feeding, one oviposition cup constructed of a piece of seed germination paper placed in a plastic 50 ml cup half full of water was set in each cage. After 1 wk (allowing for the completion of one gonotrophic cycle) the oviposition cup was replaced and the mosquitoes were allowed to feed again. At this time mortality and numbers of eggs were recorded. This process was completed

for two full gonotrophic cycles (14 d). Each paper was dried for one week before being submerged in 500 ml water with 1ml 6.0% liver powder solution for 24 hours. After this period the numbers of larvae present were counted. This experiment was repeated three times.

Due to an incubator malfunction, no eggs were observed in one of the control samples, so that sample was excluded. The same malfunction prevented collection of larval emergence data for replicate two, and those samples were excluded.

### **Spray Application**

Mosquitoes were aspirated from the maintenance colony 3 d after eclosion. Once aspirated, mosquitoes were counted and then, without being anesthetized, placed in a 50 ml plastic cup covered in bridal veil. Means of 11.2 females (range 10-15) and 14.8 males (range 10-25) were removed for each concentration. Each cup was then placed in an 18.9 liter garbage bag, where the cup was sprayed twice with a 50 ml fingertip sprayer (The Bottle Crew, West Bloomfield, MI) containing the methoprene test solution. Droplet size produced by these sprayers was  $0.17 \pm 0.04$  mm diameter, droplet density  $2.5 \pm 0.7$  droplets per  $\text{mm}^2$ , determined through the use of water sensitive paper (Teejet, Springfield, IL). During application the sprayer was held 10 cm away from the container. After application of the solution, mosquitoes were moved to wax paper cages similar to those used in experiment one. Each cage was provided sucrose soaked cotton to feed on *ad libitum*.

After 3 d each cage was provided an anesthetized mouse for feeding and an oviposition cup, and mortality was recorded. This process was completed for two full

gonotrophic cycles (14 d). The total number of larvae emerging was assessed as described for droplet application experiment, above. This experiment was repeated five times.

### **Dissections – Ovarial measurements**

Forty female mosquitoes were taken aside from the main colony 3 d after eclosion, and 10 were treated with one of the three methoprene solutions and acetone by droplet abdominal application as described above. Females were kept with 10 males for 3 d before being provided an anesthetized mouse to feed on. After 48 h, all blood-fed female mosquitoes were dissected in order to observe the effects of the solution on their ovaries.

Images were collected of the ovaries using a Leica EZ 4 HD light dissecting microscope, and processed using Leica Application Suite 2.0 software. These images were used to calculate average length of the ovaries, as well as average number of oocytes. There was a distinction in shape between treated and untreated ovarioles, and this was shown by calculating the circularity of the ovarioles. Circularity is a value between 0 (linear) and 1 (round).

## **Statistics**

Mortality of males and females was assessed using a Cox Proportional Hazards Analysis to examine significance of the differences among treatments. Kruskal-Wallis nonparametric analysis of variance was used to examine significance of the differences in fecundity among experimental treatments ( $\alpha = 0.05$ ) for fecundity measures (eggs per female and number of larvae). Pairwise comparisons were made using the Dunn method. Student's t-test and analysis of variance were performed to determine significance of the differences among treatments ( $\alpha = 0.05$ ) for all values of ovary/oocyte length, circularity, and oocyte number. All analyses were performed in JMP v.9 (SAS Institute Inc., Cary, NC).

## **Results**

### **Droplet application**

Application of a methoprene solution significantly increased female mortality ( $\chi^2=53.1$ ;  $df = 3$ ;  $P < 0.0001$ ). Adult female mosquitoes treated with the 1.0% solution experienced significantly higher mortality than the other treatments ( $P < 0.0001$ ). In males, a similar result was observed ( $\chi^2 = 44.0$ ;  $df = 3$ ;  $P < 0.0001$ ), with a significant difference between the 1.0% solution and the other treatments ( $P < 0.0001$ ; Table 2.1).

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2=0.03$ ;  $P = 0.86$ ), therefore fecundity values for both cycles were consolidated for analysis (Table 2.1). Direct application of methoprene to the abdomens of adult females had a significant effect on fecundity ( $\chi^2 = 20.7$ ;  $df = 3$ ;  $P = 0.0001$ ). Control mosquitoes produced significantly more eggs/female than mosquitoes



treated with 1.0% ( $P < 0.001$ ), 0.1% ( $P < 0.01$ ), and 0.01% ( $P = 0.04$ ) methoprene concentrations. Surviving mosquitoes treated with the 0.01% and 0.1% methoprene concentrations laid significantly more eggs than mosquitoes treated with the 1.0% methoprene concentration ( $P = 0.001$ ).

Methoprene application had a significant effect on the number of larvae resulting from the eggs of surviving treated mosquitoes ( $\chi^2 = 12.3$ ;  $df = 3$ ;  $P < 0.01$ ). Mosquito females treated with the control solution yielded significantly more larvae than all other solutions ( $P = 0.0001$ ). Fecundity values, separated by gonotrophic cycle, can be found in Table 2.1.

### **Spray application**

Spray application of methoprene has a significant effect on female mortality ( $\chi^2 = 24.1$ ;  $df = 3$ ;  $P < 0.0001$ ). Female mosquitoes treated with 1.0% methoprene spray experienced significantly higher mortality than control ( $P < 0.0001$ ), 0.01% ( $P < 0.001$ ), and 0.1% ( $P = 0.05$ ) methoprene spray treated mosquitoes. There was also a significantly higher mortality noted in the females treated with the 0.1% solution when compared with the control spray treatment ( $P = 0.01$ ). Male mosquitoes also experienced a significant difference in mortality ( $\chi^2 = 27.0$ ;  $df = 3$ ;  $P < 0.0001$ ); those treated with 1.0% methoprene spray solution experienced higher mortality than control ( $P < 0.0001$ ), 0.01% ( $P < 0.0001$ ), and 0.1% ( $P < 0.001$ ) methoprene spray treated mosquitoes (Table 2.2).

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2 = 0.19$ ;  $df = 1$ ;  $P = 0.67$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a

significant effect on fecundity ( $\chi^2 = 26.0$ ;  $df = 3$ ;  $P < 0.0001$ ). Females treated with the Control spray laid significantly more eggs than both the 0.1% methoprene treatment ( $P = 0.03$ ) and the 1.0% methoprene treatment ( $P < 0.0001$ ). Females treated with 0.01% methoprene laid significantly more eggs than those treated with 1.0% methoprene solution ( $P < 0.001$ ). The methoprene spray had a significant effect on the number of neonate larvae produced by each treated female mosquito ( $\chi^2 = 17.1$ ;  $df = 3$ ;  $P < 0.001$ ). Control females had significantly more offspring resulting from their eggs than both 0.1% ( $P < 0.01$ ) and 1.0% ( $P < 0.01$ ) methoprene solutions. The 0.01% methoprene treated females produced significantly more eggs than both the 0.1% ( $P = 0.02$ ) and 1.0% ( $P < 0.01$ ) methoprene solutions (Figure 2.2).

### **Dissections – Ovarial measurements**

Methoprene treatment caused a significant reduction in the length of adult female ovaries ( $t = 2.78$ ;  $df = 3$ ;  $P < 0.01$ ). The ovaries of female mosquitoes treated with 1.0% methoprene (Figure 2.1D) were significantly smaller than those mosquitoes treated with acetone control solution (Figure 2.1A;  $P < 0.01$ ), 0.01% methoprene (Figure 1B;  $P = 0.01$ ) and 0.1% methoprene (Figure 2.1C;  $P < 0.01$ ). Methoprene also affected length of the adult female oocytes ( $t = 2.03$ ;  $df = 3$ ;  $P < 0.0001$ ). Oocytes of the females treated with control or 1.0% solutions were significantly longer or smaller, respectively, than all other concentrations ( $P < 0.0001$ ). The 0.01% and 0.1% solutions did not significantly differ from each other ( $P = 0.28$ ). The highest dosage of methoprene (1.0%) significantly increased the number of oocytes within the ovaries of treated females relative to all other concentrations ( $t = 2.78$ ;  $df = 3$ ;  $P < 0.001$ ). Lower levels of methoprene did not affect

oocyte number (Table 2.3). Circularity of the oocytes was significantly increased by all rates of treatment with methoprene ( $t = 2.12$ ;  $df = 3$ ;  $P = 0.0001$ ; Table 2.3).

## Discussion

Under the conditions tested here, all rates of methoprene significantly reduced the fecundity of adult female mosquitoes, and the highest rate (1.0%) had a level of acute toxicity, which caused increased mortality within 3 d after treatment. Methoprene also affected the ovarian development of the female mosquitoes, reducing ovary and oocyte length, as well as increasing oocyte circularity. Treatment with methoprene caused the oocytes to remain at a small state, similar to their size before bloodfeeding. These reduced oocytes were incapable of fully maturing into eggs, and the females were unable to produce offspring. The 1.0% methoprene treatment also caused an increase in the numbers of oocytes contained within each ovary, as noted previously by Judson and Lumen (1976). In addition, those few eggs that were laid by the treated mosquitoes were less likely to hatch. This effect continued through the second gonotrophic cycle of the insect, effectively preventing the mosquito from producing offspring.

It is known that JH performs regulatory functions during vitellogenesis (Borovsky 1981). Before blood feeding, after the oocytes have reached a resting stage, JH levels drop, shortly before ecdysone is released (Noriega 2004). The results of this study could indicate a regression of the ovaries to the period of vitellogenesis, prior to the resting stage. Previous studies have noted that the length of oocytes *Ae. aegypti* mosquitoes which have entered the resting stage averaged  $0.11 \pm 0.01$ mm, increasing to  $0.31 \pm 0.02$ mm after bloodfeeding (Briegel *et al.* 2003). Ovaries of methoprene treated females

measured are similar in length to those previously recorded in the resting stage, while the oocytes of those mosquitoes treated with the control solution were capable of developing fully, indicating a regression in the ovary development in treated individuals. This regression explains the reduction in size of the oocytes, as well as the increased number of oocytes. Prior studies have noted this change, but have attributed it to the feeding habits of the treated *Ae. nigripalpus* mosquitoes. Hancock and Foster (2000) noted that mosquitoes, when treated with JH, were more likely to seek a blood meal than to feed on sugar, and that mosquitoes that consumed low amounts of sugar also exhibited lower insemination rates. This relationship led them to attribute the reduced oocyte development with the lack of a sugar meal (Hancock and Foster 2000). In the present study, mosquitoes were allowed ample time to mate and sugar feed prior to treatment, which would allow time for the ovaries to develop completely to the resting stage. This was confirmed by observation. Oocytes of treated females nevertheless regress to a smaller size even after the initial sugar meal has occurred.

For methoprene applications to play a useful role in a sex-separation strategy for mass releases, in addition to reducing female fecundity, it is a necessity that male mating performance is not affected negatively. Methoprene treatment must be able to inactivate the female reproductive systems, while not having a significant effect on male survival. The results of this study indicate that there are no significant effects on mortality caused by methoprene treatment at all but the heaviest treatment levels.

To our knowledge, the effect of JH on male mosquito mating performance has not previously been investigated. Earlier sexual maturation and enhanced pheromone release as a result of topical JH or methoprene application to tephritid fruit flies has been

demonstrated (Teal *et al.* 2000; Pereira *et al.* 2009; Haq *et al.* 2010). It is not clear why, if it occurred here, a competitive advantage would be limited to *Wolbachia*-infected males. To confirm this mating advantage, further studies should explicitly take the occurrence of multiple insemination, male body size and energetic reserves, and male accessory gland development into account.

Due to the targeted effects of methoprene on the female reproductive system, it could prove useful in the implementation of Sterile Insect Techniques (SIT). This technique aims to release into nature large numbers of sterile male insects to compete with naturally occurring males for females, potentially lowering populations of pest species (Klassen and Curtis 2005). With current methodology, all males have to be separated from the females prior to release. This is particularly important in species where the female insects transmit disease. However, no separation method for mosquitoes has proven to be absolute (Alphey 2007). By spraying 0.1% methoprene on all of the mosquitoes prior to release, there would be a reduced likelihood that any females missed during the sorting process would be capable of establishing a population. The results of this study show that the fecundity of these released females can be reduced while not reducing the survival or mating competitiveness of the released males. Extrapolations to field applications of this technique have to be made with great care due to the spatially-restrictive and carefully-controlled laboratory conditions employed. The current results support follow-up experiments in greenhouse enclosures, in which suppression scenarios using methoprene-treated males can be undertaken and the likelihood of accidentally-released females reproducing assessed under more natural conditions.

The potential of methoprene to impact adult mosquitoes also indicates that there may be applications for methoprene in the field of vector control as an air dispersed adult treatment. Methoprene is currently being applied in the field to large water sources, so there should be minimal cross contamination between a spray application of methoprene targeting adults and the treatment of water sources targeting larvae. Aerial application of methoprene to a terrestrial environment raises concern about non-target effects. Currently, aerial methoprene applications occur only in strictly controlled man-made environments (Csondes 2004). This is primarily limited to fields where flooding is used to irrigate crops, for example rice and dates. Under these conditions, the primary targets of the aerial treatment are still aquatic dipteran larvae, and effects upon terrestrial insects during these treatment conditions have not been noted. Prior studies have focused on the effects upon aquatic insects, and have not indicated any acute negative effects on either invertebrates or vertebrates (Glare and O'Callaghan 1999). However, this study does indicate a need to assess methoprene effects other than direct mortality effects. It is possible that methoprene could have long term population effects by lowering fecundity in other invertebrates.

Application of this method under controlled conditions in the field could yield important knowledge of the effects on other possible targets, as well as illuminating any complications. For instance, methoprene readily breaks down in light (Csondes 2004), and may not survive long enough in field conditions to be useful, so studies to determine the viability of methoprene as an ovicide spread by adult application are needed. Our results indicate that further research in this area is warranted.

**Table 2.1:** Mortality and fecundity measures resulting from droplet application of methoprene to the abdomens of *Ae. aegypti* mosquitoes

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
Week One	Control	16.5±9.6	22.5±6.6	4.8±2.3	1.4±2.3
	0.01%	8.7±5.0	24.7±13.3	1.5±1.07	0.04±0.04
	0.10%	14.7±16.8	32.0±13.4	0.9±0.2	0.01±0.02
	1.00%	43.0±34.5	56.5±38.3	0±0	0
Week Two	Control	34.0±10.0	44.5±14.1	3.7±3.1	0.6±0.8
	0.01%	28.7±5.3	39.4±14.0	3.7±2.5	0.02±0.03
	0.10%	41.4±19.0	46.0±4.4	1.1±0.1	0
	1.00%	64.0±13.9	69.5±6.2	0±0	0

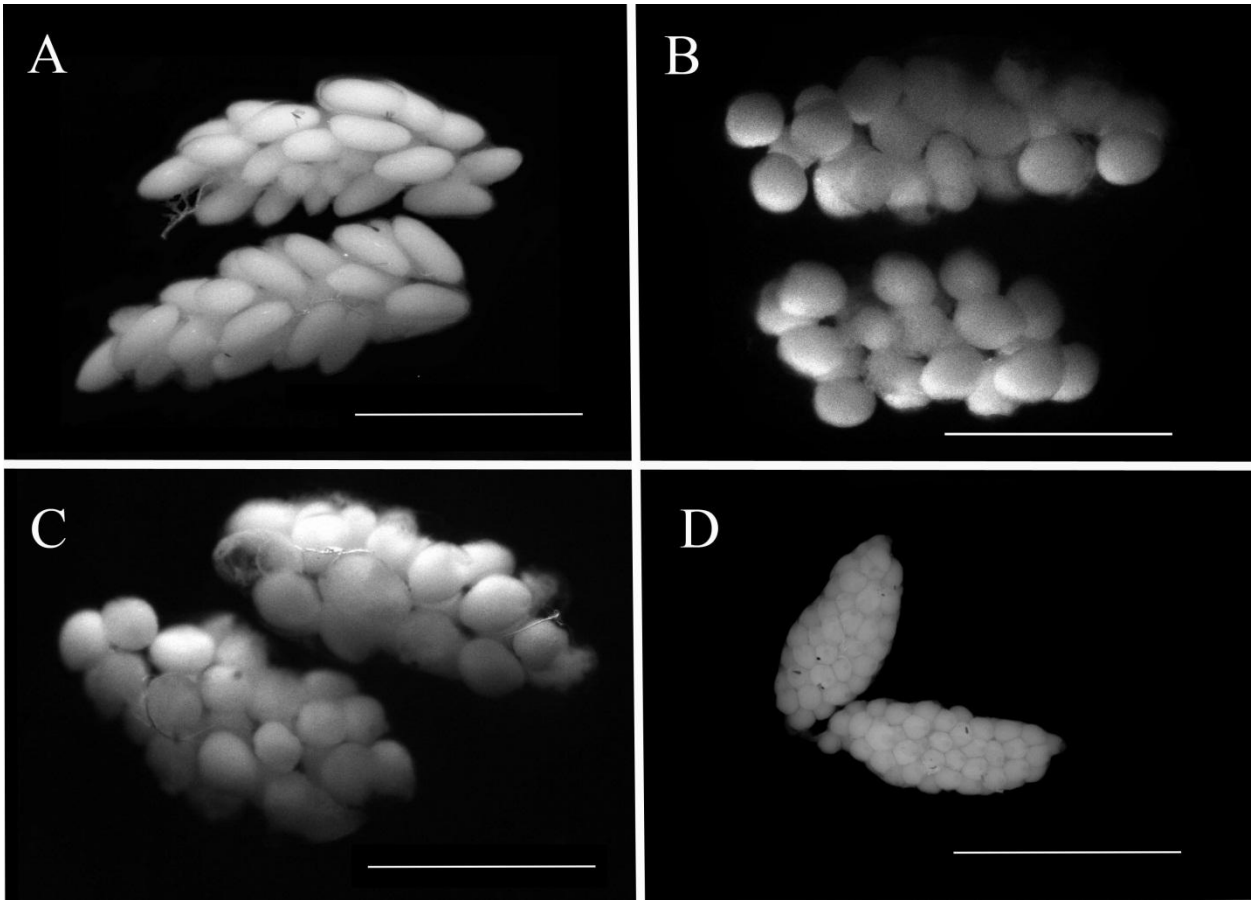
**Table 2.2:** Mortality and fecundity measures resulting from *Ae. aegypti* mosquitoes treated with methoprene spray applications

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
3 Days	Control	3.7±5.1	6.8±4.0	---	---
	0.01%	10.3±15.1	11.9±11.5	---	---
	0.1%	22.2±19.6	22.2±14.1	---	---
	1.0%	53.0±28.2	62.3±29.5	---	---
10 Days	Control	16.2±9.5	34.9±23.6	34.5±31.5	22.7±14.4
	0.01%	29.9±7.5	34.8±10.3	34.5±22.9	16.5±16.0
	0.1%	40.1±13.9	42.5±9.8	3.2±5.3	3.1±3.8
	1.0%	67.9±11.3	80.8±13.0	0±0	0.0±0.1
17 Days	Control	35.0±19.6	54.9±16.4	60.5±30.8	21.6±14.5
	0.01%	40.5±11.7	51.2±12.5	51.3±31.6	14.3±7.3
	0.1%	61.0±18.1	56.3±15.3	11.8±13.9	5.0±5.3
	1.0%	73.4±12.2	86.0±5.5	10±22.4	0.5±1.0

**Table 2.3:** Length of ovaries as well as numbers, circularity, and length of oocytes calculated from dissections of *Ae. aegypti* mosquitoes

<b>Treatment</b>	<b>Ovary length (mm)</b>	<b>Oocyte length (mm)</b>	<b>No. of oocytes</b>	<b>Circularity</b>
<b>Control</b>	1.76±0.08	0.38±0.02	20.5±0.71	0.71±0.03
<b>0.01%</b>	1.73±0.28	0.29±0.03	22.5±6.36	0.89±0.03
<b>0.10%</b>	1.79±0.06	0.28±0.03	22.0±1.41	0.88±0.02
<b>1.00%</b>	1.01±0.08	0.11±0.02	39.5±2.12	0.89±0.05





**Figure 2.1:** *Ae. aegypti* ovaries treated with methoprene. Ovaries were removed 48 hours post-bloodmeal. A) Ovary treated with acetone control B) 0.01% methoprene treated ovary C) 0.1% methoprene treated ovary D) 1.0% methoprene treated ovary. Scale bar is equivalent to 1mm.

## Chapter 3

### Effects of methoprene on adult *Aedes albopictus* and *Aedes polynesiensis* mosquitoes

#### Introduction

Previously, methoprene was applied, both by droplet and spray application, to adult *Ae. aegypti* mosquitoes. Application of methoprene caused a reduction in the size of female oocytes and a significant drop in fecundity. There were significant effects on mortality noted at high concentrations of methoprene, but no significant differences at lower treatment levels. The effect on reproduction was hypothesized to be connected to the appearance of the mosquito ovaries that had been arrested in a “resting stage” approximately 100  $\mu\text{m}$  in length (Chapter 2).

The observation in Chapter 2, that methoprene has a greater effect on females relative to males, has a potential applied use. If applied to control methods based on mass releases of male mosquitoes, pretreatment of the release material with methoprene could have relatively little effect on the males while diminishing the potential fecundity any unintentionally released females. Examples include SIT strategies in which *Wolbachia* infected male mosquitoes are released for population suppression. This approach is based on *Wolbachia*-induced cytoplasmic incompatibility and is being developed for various mosquito species (Atyame *et al.*, 2011).

The purpose of this study was to assess the differences in fecundity and mortality measures, if any, in treatment effects of methoprene on other members of the genus *Aedes*. By analyzing these differences, the authors hope to gain an understanding of the

capabilities of methoprene as a method of reducing female mosquito reproductive success. The two species chosen for this study were chosen for specific reasons.

*Ae. albopictus*, the Asian Tiger Mosquito, is an important invasive pest which has become prevalent in the United States. It is a highly anthropophilic day feeding mosquito which is competent vector of many human and animal diseases. *Ae. albopictus* is a capable vector for Dengue, West Nile, LaCrosse, and Chikungunya viruses, and also Dog Heartworm (Juliano and Lounibos, 2005). This mosquito has dramatically increased in range recently, helped by the international trade of tires, which can contain mosquito eggs (Reiter 1998). Studies have illustrated that *Ae. albopictus* can already be found in over 26 countries, and has the potential to become globally cosmopolitan (Benedict *et al*, 2007). Control of *Ae. albopictus* is difficult, due to the tendency of the species to use small, hard to find sources of water. An aerial application of methoprene has the potential to reach adult *Ae. albopictus* mosquitoes in urban environments that would otherwise be difficult to successfully treat with insecticide.

*Ae. polynesiensis*, the Polynesian Tiger Mosquito, is an important vector of Lymphatic Filariasis in the South Pacific (Nicholas and Scoles, 1997). *Ae. polynesiensis* are diurnal and feeding peaks at dawn and dusk (Russell 2004). As with other *Aedes* species, vector control is complicated due to the small containers used by *Ae. polynesiensis* (Lardeux *et al*, 2002). Recent work with this pest has focused on the release of incompatible male mosquitoes for population control (Brelsfoard *et al*, 2008).

Pesticide exposure also differs between the species and strains utilized. *Ae. aegypti* mosquitoes previously analyzed were members of the Liverpool strain, colonized

prior to the invention of methoprene (Macdonald 1962). Consequently, it is possible that results received using a long maintained laboratory colony might be different than effects on wild populations. *Ae. albopictus* used for this study were collected from an existing wild population in order to show the applicability of methoprene as a treatment against established populations of mosquitoes. Application of methoprene to *Ae. albopictus* in this study is to illustrate the effects of a methoprene treatment on wild populations of mosquitoes. This will illustrate the practicality of methoprene as a spray treatment under field conditions.

In contrast, mosquitoes in the South Pacific area have had less exposure to insect growth regulators. Since the advent of methoprene in 1972, applications of methoprene in the South Pacific region have increased, though not to the levels used in the continental US (Russell and Kay 2004). *Ae. polynesiensis* used in this study were from a laboratory raised colony of potential *Wolbachia* biopesticide control mosquitoes. These mosquitoes have been raised with a novel infection of *Wolbachia* bacterium for use in mass release and population suppression (Brelsfoard *et al*, 2007). Also, due to the environmental constraints of using pesticides in an island habitat, methoprene is primarily used within cities (Wood *et al*, 2007). Water treatment does not assist with the control of *Ae. polynesiensis*, due to the number of smaller, natural water sources used as breeding sites in island habitats (Rosen *et al*, 1954).

## Methods

### Methoprene

One gram of 99.97% technical grade methoprene was obtained from Central Life Sciences (Dallas, Texas) for use during this study. For this study, 50 mL concentrations of 1%, 0.1%, 0.01% and 0.001% methoprene were created by blending technical grade methoprene with acetone. The control solution was acetone alone.

### Mosquitoes

Two species of *Aedes* mosquitoes were used in this study. *Ae. albopictus* mosquitoes belonged to the WC strain, which was collected wild in Lexington, Kentucky during the summer of 2011 and established as a laboratory colony. *Ae. polynesiensis* mosquitoes belonged to the CP strain (Brelsfoard *et al*, 2007). The colonies were maintained as previously described (Chapter 2). In brief, seed germination paper (Anchor Paper, Saint Paul, Minnesota) which mosquitoes had oviposited on were submerged in 500 ml water and emergent larvae were reared in optimal conditions with the daily addition of food (6.0% liver powder solution). All mosquitoes used in this study were kept at 27°C and 70% RH, with a 14:10 hour light: dark cycle inside an incubator (Model I-36VL, Percival Scientific, Perry, IA). Cages of adults were provided constantly with a 10% sucrose solution.

## **Droplet Application**

Mosquitoes were aspirated from the maintenance colony three days after pupation using a motorized aspirator, and then anesthetized with chloroform for 30 seconds. Each mosquito was placed ventral side up using soft forceps, and 0.5  $\mu$ L of test solution was applied directly to the abdomen using an Eppendorf 2  $\mu$ L micropipette (Hamilton; Reno, Nevada). Twenty dosed male and female mosquitoes were then moved into each of five cages (one for each experimental concentration, as well as one control cage treated with acetone) constructed of 83oz wax paper buckets (Solo Cup Company; Lake Forest Illinois) with a sleeve attached to the side constructed of medical stockinette (Dynarex; Orangeburg, New York). Mosquitoes were given an hour to recover from the chloroform before sucrose wicks were provided.

Three days after dosing, mosquitoes were provided one anesthetized mouse (IACUC #00905A2005) to feed on for 15 minutes. After feeding, one oviposition cup constructed of a piece of seed germination paper (Anchor paper company) placed in a cup half full of water was set in each cage. After one week (allowing for the completion of one gonotrophic cycle) the oviposition cup was replaced and the mosquitoes were allowed to feed again. At this time mortality and numbers of eggs was recorded. This process was completed for two full gonotrophic cycles (14 d). Each paper was dried for one week before being submerged in 500 mL water with 1mL 6.0% liver powder solution for 24 hours. After this period the numbers of larvae present were counted in order to assess the number of larvae emerging per adult female mosquito. This experiment was repeated three times for *Ae. polynesiensis*. This experiment was not performed on *Ae. albopictus*, as the applications of methoprene consumed a significant amount of time.

## **Spray Application**

Mosquitoes were aspirated from the maintenance colony three days after eclosion. Once aspirated, mosquitoes were counted and then, without being anesthetized, placed in a small plastic cup covered in bridal veil. Approximately 10 females and 10 males were removed for each concentration. Each cup was then placed in a 18.9 L garbage bag to prevent the spread of methoprene from the testing area, where the cup was sprayed twice with a 50 ml fingertip sprayer (The Bottle Crew; West Bloomfield, Michigan) containing the methoprene test solution. Droplet size produced by these sprayers was  $0.17 \pm 0.04$  mm, droplet density  $2.5 \pm 0.7$  droplets per  $\text{mm}^2$ , determined through the use of water sensitive paper (Teejet; Springfield, Illinois). During application the sprayer was held 10 cm away from the container. After application of the solution, mosquitoes were moved to wax paper cages similar to those used in experiment one. Each cage was provided a sucrose soaked wick to feed on ad libitum.

After three days each cage was provided an anesthetized mouse for feeding and an oviposition cup, and mortality was recorded. This process was completed for two full gonotrophic cycles (14 d). The total number of larvae emerging was assessed as described for the droplet application, above. This experiment was repeated five times.

## **Dissections – Ovarial measurements**

Forty female mosquitoes were separated from the main colony three days after eclosion, and 10 were treated with one of the four experimental methoprene solutions by direct abdominal application similar to experiment one. Females were kept with 10 males for three days before being provided an anesthetized mouse to feed on. All

mosquitoes that did not feed were then removed. After 48 hours all blood-fed female mosquitoes were dissected in order to observe the effects of the solution on their ovaries.

Images were collected of the ovaries using a Leica EZ 4 HD light dissecting microscope, and processed using Leica Application Suite 2.0 software. These images were used to calculate average length of the ovaries, as well as average number of oocytes. There was a distinction in shape between treated and untreated ovarioles, and this was shown by calculating the circularity of the ovarioles. Circularity is a value between 0 and 1 which indicates how close a particle is to perfectly round. When this value is 1, the particle is perfectly round.

## Results

### Droplet application

Application of a methoprene solution had a significant effect on *Ae. polynesiensis* female mortality ( $\chi^2=111.8$ ;  $df = 3$ ;  $P < 0.0001$ ). Adult female mosquitoes treated with the 1.0% solution experienced significantly higher mortality than the other treatments ( $P < 0.0001$ ; Table 3.1). Also, female mosquitoes treated with the control solution experienced a significantly lower mortality than all other treatments ( $P < 0.0001$ ). In males, a similar result was observed ( $\chi^2 = 57.10$ ;  $df = 3$ ;  $P < 0.0001$ ), with significantly higher mortality between the 1.0% solution and the other treatments ( $P < 0.0001$ ; Table 3.1), and a significantly lower mortality between the control and all other treatments ( $P < 0.0001$ ; Table 3.1).

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2 = 0.08$ ;  $P = 0.8$ ), therefore fecundity values for both



cycles were combined for analysis (Table 3.1). Direct application of methoprene to the abdomens of adult females had a significant effect on fecundity ( $\chi^2 = 16.4$ ;  $df = 3$ ;  $P = 0.001$ ). Control mosquitoes produced significantly more eggs/female than mosquitoes treated with 1.0% ( $P = 0.003$ ) and 0.1% ( $P = 0.003$ ), but not significantly more than those mosquitoes treated with 0.01% ( $P = 0.06$ ) methoprene concentrations. There were no other significant relationships in egg production (Table 3.1).

Methoprene application had a significant effect on the number of larvae resulting from the eggs of treated mosquitoes ( $\chi^2 = 10.2$ ;  $df = 3$ ;  $P = 0.02$ ). Mosquito females treated with the control solution yielded significantly more larvae than than mosquitoes treated with 1.0% ( $P = 0.03$ ) and 0.1% ( $P = 0.03$ ), but not significantly more than those mosquitoes treated with 0.01% ( $P = 0.13$ ) methoprene concentrations. Fecundity values, separated by gonotrophic cycle, can be found in Table 3.1.

### **Spray application**

#### *Aedes polynesiensis*

Spray application of methoprene has a significant effect on *Ae. polynesiensis* female mortality ( $\chi^2 = 39.4$ ;  $df = 3$ ;  $P < 0.0001$ ). Female mosquitoes treated with 0.1% methoprene spray experienced significantly higher mortality than control ( $P < 0.0001$ ), 0.001% ( $P < 0.0001$ ), but was not significantly higher than those mosquitoes treated with 0.01% ( $P = 0.5$ ) methoprene spray. There was also a significantly higher mortality noted in the females treated with the 0.01% solution when compared with the control spray treatment ( $P < 0.0001$ ) and the 0.001% solution ( $P = 0.0005$ ). Male *Ae. polynesiensis* mosquitoes also experienced a significant difference in mortality ( $\chi^2 = 20.77$ ;  $df = 3$ ;  $P =$

0.0001). Male mosquitoes treated with 0.1% methoprene spray solution experienced significantly higher mortality than control ( $P < 0.0001$ ), 0.001% ( $P < 0.001$ ), and 0.01% ( $P = 0.02$ ) methoprene spray treated mosquitoes. Male mosquitoes treated with 0.01% methoprene spray also experienced significantly higher mortality than mosquitoes treated with control spray ( $P = 0.02$ ; Table 3.2).

There were no significant differences in fecundity values between the two observed gonotrophic cycles for *Ae. polynesiensis* ( $\chi^2 = 0.2$ ;  $P = 0.7$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a significant effect on fecundity ( $\chi^2 = 24.63$ ;  $df = 3$ ;  $P < 0.0001$ ). Females treated with the Control spray laid significantly more eggs than both the 0.01% methoprene treatment ( $P = 0.003$ ) and the 0.1% methoprene treatment ( $P < 0.001$ ). Females treated with 0.001% methoprene laid significantly more eggs than those treated with 0.01% methoprene solution ( $P = 0.0019$ ) and those treated with the 0.1% methoprene solution ( $P = 0.0006$ ). Females treated with 0.01% methoprene laid significantly more eggs than those treated with 0.1% methoprene solution ( $P = 0.0025$ ). The methoprene spray had a significant effect on the number of first instar larvae produced by each treated female mosquito ( $\chi^2 = 22.30$ ;  $df = 3$ ;  $P < 0.0001$ ). Control females had significantly more offspring resulting from their eggs than both 0.01% ( $P = 0.005$ ) and 0.1% ( $P = 0.002$ ) methoprene solutions. The 0.001% methoprene treated females were capable of laying significantly more eggs than both the 0.01% ( $P = 0.001$ ) and 0.1% ( $P = 0.004$ ) methoprene solutions. Specific values can be found in Table 3.2.

*Aedes albopictus*

Spray application of methoprene has a significant effect on *Ae. albopictus* female mortality ( $\chi^2 = 80.2$ ;  $df = 3$ ;  $P < 0.0001$ ). Female mosquitoes treated with 1.0% methoprene spray experienced significantly higher mortality than control ( $P < 0.0001$ ), 0.01% ( $P < 0.0001$ ), and 0.1% ( $P < 0.0001$ ) methoprene spray. There was also a significantly higher mortality noted in the females treated with the 0.1% solution when compared with the control spray treatment ( $P = 0.007$ ) and the 0.01% solution ( $P = 0.03$ ). Male *Ae. albopictus* mosquitoes also experienced a significant difference in mortality ( $\chi^2 = 86.9$ ;  $df = 3$ ;  $P < 0.0001$ ). Male mosquitoes treated with 1.0% methoprene spray solution experienced significantly higher mortality than control ( $P < 0.0001$ ), 0.001% ( $P < 0.0001$ ), and 0.01% ( $P < 0.0001$ ) methoprene spray treated mosquitoes. No other significant mortality effects were noted (Table 3.3).

There were no significant differences in fecundity values between the two observed gonotrophic cycles for *Ae. albopictus* ( $\chi^2 = 2.1$ ;  $P = 0.2$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a significant effect on fecundity ( $\chi^2 = 9.0$ ;  $df = 3$ ;  $P = 0.04$ ). Females treated with the 1.0% methoprene spray laid significantly fewer eggs than the control ( $P = 0.02$ ), 0.01% ( $P = 0.04$ ), and 0.1% ( $P = 0.04$ ) methoprene treatments (Table 3.3). The methoprene spray had a significant effect on the number of first instar larvae produced by each treated female mosquito ( $\chi^2 = 18.9$ ;  $df = 3$ ;  $P < 0.001$ ). Control females had significantly more offspring resulting from their eggs than 0.01% ( $P = 0.04$ ), 0.1% ( $P = 0.008$ ), and 1.0% ( $P = 0.003$ ) methoprene solutions. The 1.0% methoprene treated females were capable of

laying significantly less eggs than both the 0.01% ( $P = 0.003$ ) and 0.1% ( $P = 0.003$ ) methoprene solutions. Specific values can be found in Table 3.3.

### **Dissections- Ovarial measurements**

#### *Aedes polynesiensis*

Methoprene treatment has no significant effect on the length of adult female ovaries ( $t = 2.78$ ). The length of the adult female oocytes were significantly affected by methoprene treatment ( $t = 2.03$ ). The oocytes of female mosquitoes treated with the control solution were significantly larger than those treated with the 0.01% ( $P = 0.02$ ) and 0.1% ( $0.19 \pm 0.10$ mm;  $P < 0.0001$ ) solutions, however was not significantly larger than oocytes of mosquitoes treated with the 0.001% solution ( $P = 0.16$ ). Oocytes of mosquitoes treated with 0.1% methoprene solution were significantly smaller than 0.01% ( $P = 0.003$ ) and 0.001% ( $P = 0.0001$ ) treated mosquitoes. Methoprene treatment had a significant effect on the numbers of oocytes contained within the ovaries ( $t = 2.78$ ). There were significantly more oocytes found in those mosquitoes that have been treated with 0.1% methoprene when compared with control ( $P = 0.04$ ) and 0.001% ( $P = 0.02$ ) methoprene solutions. There were no more significant oocyte number relationships. Circularity was also significantly affected by treatment with methoprene ( $t = 2.12$ ). The oocytes of the 0.1% methoprene treated mosquitoes were significantly higher in circularity than those mosquitoes treated with Control ( $P < 0.0001$ ), 0.001% ( $P = 0.03$ ), and 0.01% ( $P = 0.0002$ ) methoprene solutions. Mosquitoes treated with 0.001% methoprene had oocytes which were significantly higher in circularity than 0.01% ( $P =$

0.03) and control ( $P= 0.005$ ) solutions. Exact values are shown in Table 3.4 and images of the dissected ovaries can be observed in Figure 3.1.

### *Aedes albopictus*

Methoprene treatment has no significant effect on the length of adult female ovaries ( $t = 2.78$ ). The length of the adult female oocytes were significantly affected by methoprene treatment ( $t = 2.03$ ). The oocytes of female mosquitoes treated with 1.0% methoprene solution were significantly smaller than Control ( $P= 0.0001$ ), 0.01% ( $P= 0.001$ ), and 0.1% ( $P < 0.0001$ ) methoprene solutions. Methoprene treatment also had a significant effect on the numbers of oocytes contained within the ovaries ( $t = 2.78$ ).

There were significantly more oocytes found in those mosquitoes treated with the control solution than those treated with 0.1% ( $P= 0.04$ ) and 1% ( $P= 0.03$ ) methoprene solutions, and no significant difference with the 0.01% ( $P= 0.62$ ) treatment. Mosquitoes treated with 1% methoprene had significantly less oocytes than those treated with Control ( $P= 0.03$ ) and 0.01% ( $P= 0.05$ ) methoprene solutions, and no significant difference from the 0.1% methoprene solution ( $P= 0.7$ ). Circularity was also significantly affected by treatment with methoprene ( $t = 2.12$ ). The oocytes of the mosquitoes treated with 1.0% methoprene solution were significantly higher in circularity than those mosquitoes treated with 0.1% ( $P = 0.03$ ) methoprene solution. There were no other significant relationships. Exact values are shown in Table 3.5 and images of the dissected ovaries can be observed in Figure 3.2.

## Discussion

The results indicated by this study are similar to those results received previously with *Ae. aegypti*. Application of methoprene caused the oocytes of female mosquitoes to develop poorly, and lowered the fecundity of treated mosquitoes. Mortality was also significantly affected. Higher concentrations of methoprene caused significant mortality in both species tested. However, there were also marked differences.

In *Ae. polynesiensis*, there appeared to be a much lower level of methoprene needed in order to achieve acute toxicity. Mosquitoes treated with 1.0% methoprene did not survive long enough for fecundity to be appraised. 100% mortality was reached within 24 hours after treatment, too soon for any ovarial development to be determined visually. Instead all treatments were reduced by a factor of ten, allowing observation of methoprene effects on ovarial development without reaching significant levels of acute mortality. In both the droplet and spray treatments mortality was higher in comparison to previous work with *Ae. aegypti* and currently with *Ae. albopictus*. This can likely be attributed to the weakness of the species as a whole. Mosquitoes treated with the control spray still experienced heavy mortality and a reduced fecundity in comparison with the *Ae. albopictus* and *Ae. polynesiensis*. Treatment of the mosquitoes using a 0.1% methoprene solution was shown to be able to completely remove the ability of the mosquito to produce offspring, and produce results similar to those found with *Ae. aegypti* females treated with 1.0% methoprene solution.

*Ae. albopictus* mosquitoes seemed to be able to tolerate methoprene treatment more easily than *Ae. polynesiensis* and *Ae. aegypti*. At lower levels of methoprene

treatment, it does not seem that methoprene had any significant effect on adult mosquito health. There did appear to be a level of acute toxicity associated with 1.0% methoprene treatment. This treatment caused massive level of mortality and appeared to be the only level of treatment which significantly affects the numbers of eggs produced. While methoprene treatment of *Ae. albopictus* did not have a significant effect on egg production, it did greatly reduce the amount of neonate larvae which resulted from those eggs. Treatment of the mosquito with any level of methoprene causes a significant reduction in larval number. Ovarial dissections of the mosquitoes show a potential explanation.

Not all members of the genus have the same reproductive biology. *Ae. albopictus* and *Ae. polynesiensis* mosquitoes have a 24 hour longer oocyte development time than *Ae. aegypti* (Personal observations). In these mosquitoes dissections had to take place 72 hours post bloodfeeding to observe the same state of ovarian development which was visible in *Ae. aegypti* after 48 hours (Brabant, unpublished findings). In these mosquitoes there appears to be a secondary effect of methoprene exposure. It was previously noted that exposure to methoprene caused reduction in the size of the ovaries, and an increase in circularity of the oocytes. While this effect is present in *Ae. polynesiensis*, it is not as dramatic an effect as in *Ae. aegypti*. What is readily visible is a “white fog” rising off the individual oocytes which was not previously noted in *Ae. aegypti*. This fog appears to be the yolk of the oocytes being released into the dissecting solution. This effect is more pronounced with higher levels of methoprene. This could hypothetically represent the weakening of the chorion of the developing insect oocyte. This weakened shell would be less likely to hold in nutrients, and would be not be able to support any eggs which were

capable of being laid. In *Ae. albopictus* this effect is more pronounced. There is no reduction in the size of the oocytes, but there is a cloud rising from the edge of each developing egg. This cloud is the yolk of the oocytes being released, and increases in presence for each level of methoprene exposure.

While the physiological responses within these two mosquito species differ, the phenotypic response is the same. Methoprene causes a significant effect on the female mosquito to produce offspring. This shows that methoprene treatment could be a viable method for controlled release of mosquitoes in sterile male release scenarios. Treatment with methoprene prior to release can lower female fecundity to negligible levels, while not effecting male mortality. Future studies can be performed to determine if methoprene has any further effects on male virility or reproductive development.



**Table 3.1:** Mortality and fecundity measures involving droplet application of methoprene to the abdomen of *Ae. polynesiensis*

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
<b>3 Days</b>	<b>Control</b>	18.5±7.5	33.5±3.0	---	---
	<b>0.01%</b>	28.5±15.5	68.5±12.5	---	---
	<b>0.10%</b>	46.5±16.0	78.5±16.0	---	---
	<b>1.00%</b>	96.5±6.0	100.0±0.0	---	---
<b>10 Days</b>	<b>Control</b>	45.0±15.0	73.5±7.5	---	---
	<b>0.01%</b>	63.5±29.5	91.5±6.0	---	---
	<b>0.10%</b>	78.5±20.0	96.5±3.0	---	---
	<b>1.00%</b>	100.0±0.0	100.0±0.0	---	---
<b>17 Days</b>	<b>Control</b>	65.0±20.0	86.5±10.5	20.4±15.8	9.6±3.9
	<b>0.01%</b>	93.5±7.5	98.5±3.0	12.4±5.1	2.2±5.4
	<b>0.10%</b>	90.0±10.0	98.5±3.0	0.0±0.0	0.0±0.0
	<b>1.00%</b>	100.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0

**Table 3.2:** Mortality and fecundity measures involving spray application of methoprene to *Ae. polynesiensis*

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
<b>3 Days</b>	<b>Control</b>	17.8±13.2	28.5±17.8	---	---
	<b>0.001%</b>	16.1±14.5	33.0±11.5	---	---
	<b>0.01%</b>	12.7±9.8	17.2±8.4	---	---
	<b>0.1%</b>	45.3±36.7	67.6±34.0	---	---
<b>10 Days</b>	<b>Control</b>	41.0±9.2	56.1±27.0	---	---
	<b>0.001%</b>	43.7±25.5	61.0±5.9	---	---
	<b>0.01%</b>	60.3±26.6	66.1±14.9	---	---
	<b>0.1%</b>	76.7±24.6	81.3±28.4	---	---
<b>17 Days</b>	<b>Control</b>	56.3±13.3	70.8±23.5	27.8±18.2	19.4±14.6
	<b>0.001%</b>	62.0±14.0	81.1±16.0	19.7±18.5	9.5±11.4
	<b>0.01%</b>	85.4±13.9	90.7±8.1	3.2±2.8	0.5±0.9
	<b>0.1%</b>	84.2±18.1	89.7±14.2	0.04±0.1	0.5±1.0

**Table 3.3:** Mortality and fecundity measures involving spray application of methoprene *Ae. albopictus*

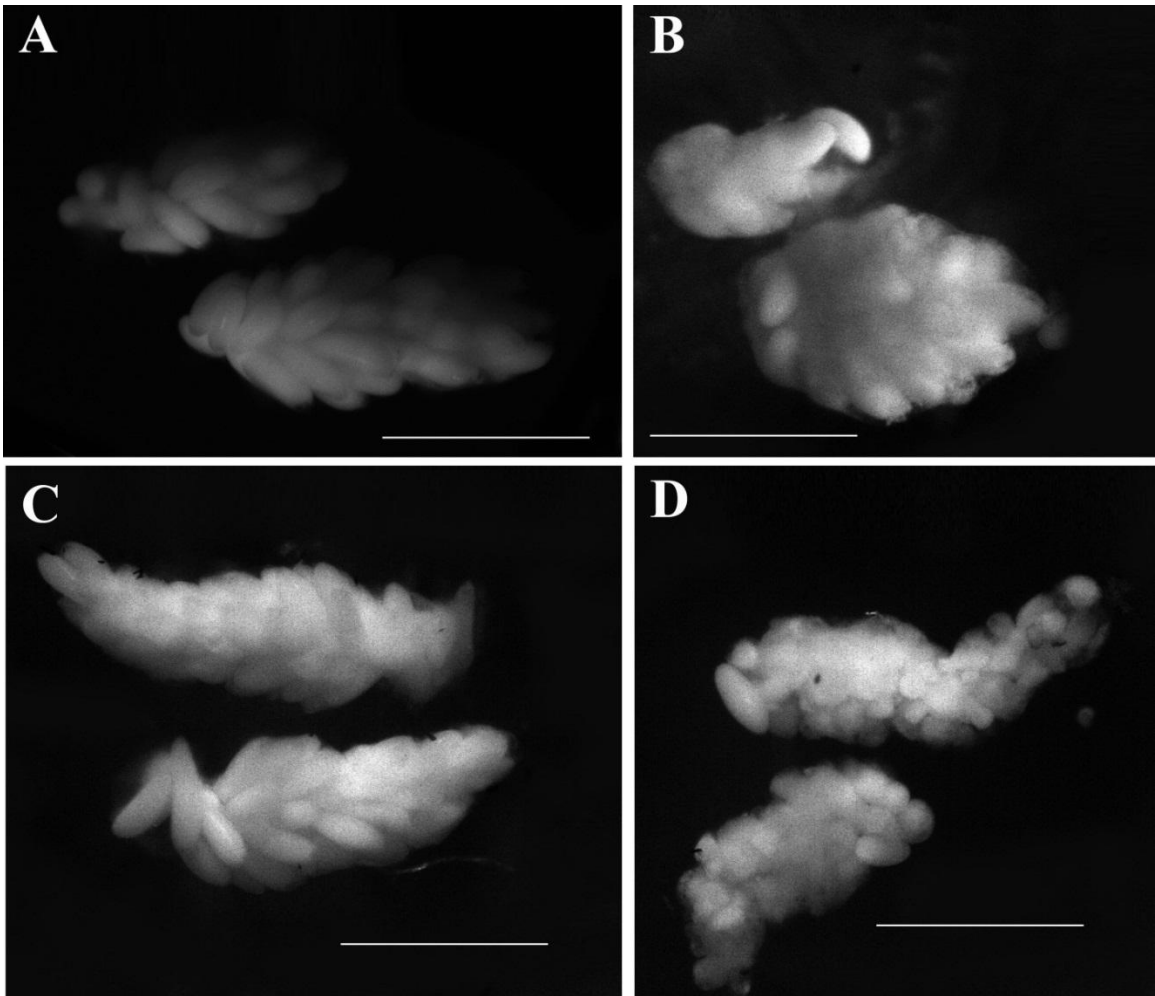
Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
3 Days	Control	0±0.0	3.0±5.3	---	---
	0.01%	20.2±29.0	21.9±13.0	---	---
	0.1%	22.0±6.6	34.6±18.6	---	---
	1.0%	85.3±15.4	98.2±3.0	---	---
10 Days	Control	21.2±18.9	71.6±10.6	---	---
	0.01%	22.5±27.6	55.6±10.6	---	---
	0.1%	40.2±19.5	74.8±16.3	---	---
	1.0%	90.4±8.4	98.2±3.0	---	---
17 Days	Control	24.2±22.3	90.4±4.4	37.4±21.4	29.9±17.9
	0.01%	30.0±31.9	76.3±6.1	28.2±16.0	11.6±7.9
	0.1%	46.3±22.3	76.7±16.2	19.7±14.4	4.4±7.3
	1.0%	95.2±4.2	100.0±0.0	8.6±17.2	0.0±0.0

**Table 3.4:** Length of ovaries as well as numbers, circularity, and length of oocytes calculated from dissections performed on *Ae. polynesiensis*

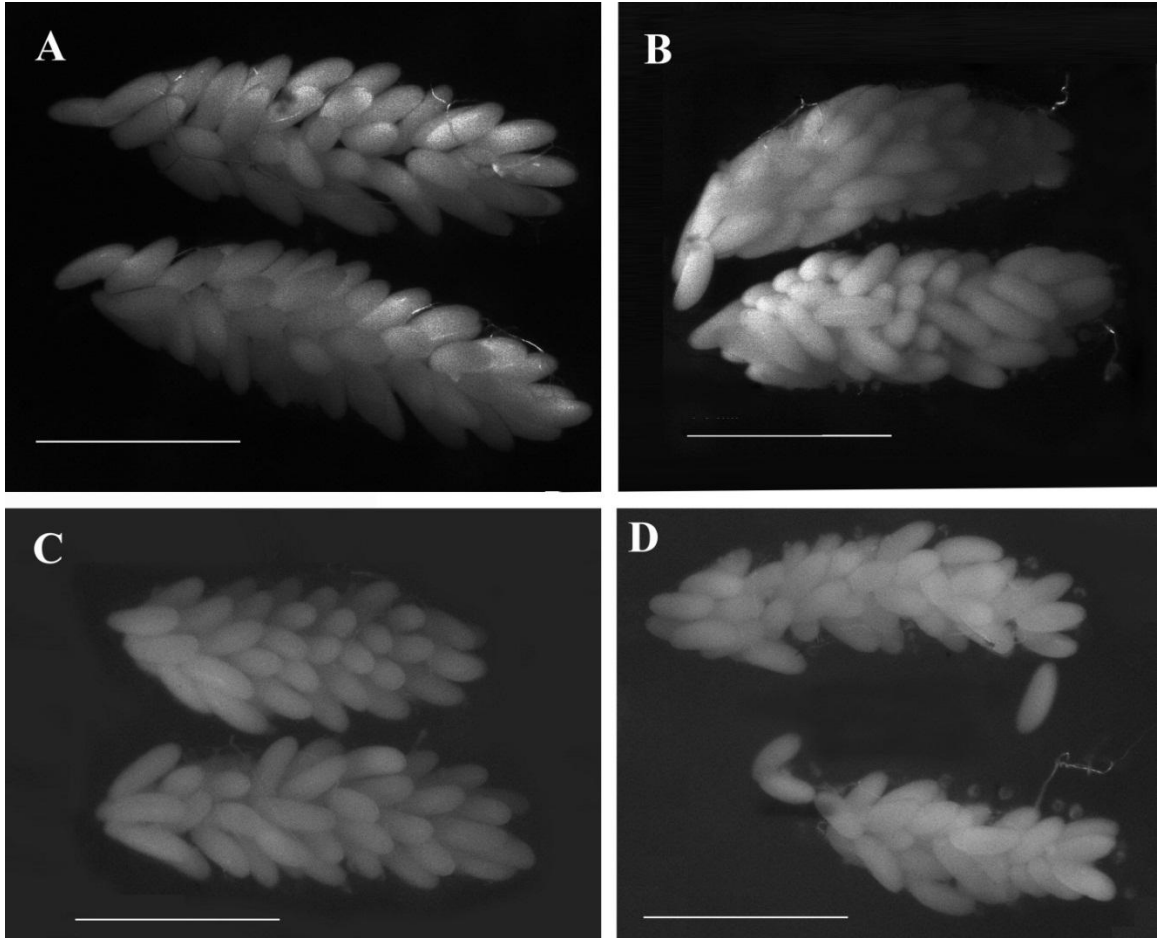
Treatment	Ovary Length (mm)	Oocyte Length (mm)	No. of oocytes	Circularity
Control	1.45±0.37	0.36±0.04	27.0±8.4	0.62±0.05
0.001%	1.25±0.35	0.31±0.06	17.5±10.6	0.76±0.04
0.01%	1.93±0.21	0.28±0.06	41.5±5.0	0.67±0.09
0.1%	1.84±0.46	0.19±0.10	59.0±17.0	0.85±0.02

**Table 3.5:** Length of ovaries as well as numbers, circularity, and length of oocytes calculated from dissections performed on *Ae. albopictus*

Treatment	Ovary Length (mm)	Oocyte Length (mm)	No. of oocytes	Circularity
Control	2.33±0.14	0.41±0.02	46.5±2.12	0.70±0.05
0.01%	1.96±0.07	0.40±0.02	44.5±0.71	0.69±0.03
0.1%	1.92±0.25	0.41±0.02	35.5±0.71	0.65±0.03
1.0%	1.73±0.46	0.36±0.03	34.0±7.07	0.72±0.04



**Figure 3.1:** *Ae. polynesiensis* ovaries treated with methoprene. Ovaries were removed 72 hours post-bloodmeal. A) Ovary treated with acetone control B) 0.001% methoprene treated ovary C) 0.01% methoprene treated ovary D) 0.1% methoprene treated ovary. Scale bar is equivalent to 1mm.



**Figure 3.2:** *Ae. albopictus* ovaries treated with methoprene. Ovaries were removed 72 hours post-bloodmeal. A) Ovary treated with acetone control B) 0.01% methoprene treated ovary C) 0.1% methoprene treated ovary D) 1.0% methoprene treated ovary. Scale bar is equivalent to 1mm.

## Chapter 4

### Effects of Altosid® on female and male *Aedes* mosquitoes

#### Introduction

Prior research has shown that methoprene treatment has a significant effect on larval mortality (Norland and DeWitt 1975). In prior chapters, treatment with 1% methoprene causes an immediate drop off in survivorship of mosquitoes, killing many within 3 d after treatment. This acute mortality was found in both male and female mosquitoes. In order for methoprene treatment to be used with the *Wolbachia* bio-pesticide approach, male mortality must be reduced to a minimum. Therefore a less severe method of application is required, where fecundity effects remain significant, while mortality effects are minimized.

Altosid® is a commercially available pesticide which functions as an insect growth regulator. It contains a proprietary blend of chemicals, with the active ingredient methoprene, which functions by mimicking Juvenile Hormone (JH) as previously described in chapter two. This pesticide is often applied to man-made water sources to control nematoceran Diptera, and vector control districts will often use Altosid® to control mosquito larvae (Boxmeyer *et al.* 1997). Altosid® is formulated for water application, and is not known to function against adult mosquitoes (Csondes 2004). The commercial formulation of Altosid® contains 5.0% methoprene by volume, which is blended with other constituents to enhance dissolution of the pesticide in water. These chemicals are inert, and are not known to affect insect populations in any way (Norland and DeWitt 1975). As a commercially available insecticide, Altosid® is more easily

obtained than technical grade methoprene, and easily blends with water for simple dilutions.

For these reasons, Altosid® was chosen for assessment as a simple methoprene application. The purpose of this study was to assess the ability of Altosid® to reduce the fecundity of adult female mosquitoes while minimizing effects on male mosquitoes, and to compare the mortality effects of Altosid® on three different species of mosquitoes within the same genus. This information would prove useful for those working to control mosquitoes in the field, in addition to laboratory settings. For example, Altosid® could be shipped easily across the ocean to a laboratory preparing to mass release *Wolbachia* infected males, and once there, could be blended with water and applied to mosquitoes that have been isolated prior to release. Ideally, this application would reduce or eliminate the fecundity of any female mosquitoes that were not removed during the sorting process, and make it safe to release the males.

## **Methods**

### **Mosquitoes**

Three species of mosquitoes were used in this study. *Ae. aegypti* used in this study were the Liverpool strain, obtained from the NIAID/NIH Filariasis Research Reagent Resource Center (FR3) in June 2009. *Ae. albopictus* mosquitoes belonged to the WC strain, which was collected wild in Lexington, Kentucky during the summer of 2011 and established as a laboratory colony. *Ae. polynesiensis* mosquitoes belonged to the CP strain (Brelsfoard *et al*, 2007). The colonies were maintained as previously described in chapter two. In brief, seed germination paper (Anchor Paper, Saint Paul, MN) which

mosquitoes had oviposited on were submerged in 500 ml water, and emergent larvae were reared in optimal conditions with the daily addition of food (6.0% liver powder solution). All mosquitoes used in this study were kept at 27°C and 70% RH, with a 14:10 hour light: dark cycle inside an incubator (Model I-36VL, Percival Scientific, Perry, IA). Cages of adults were provided constantly with a 10% sucrose solution.

### **Spray Application**

Mosquitoes were aspirated from the maintenance colony three days after eclosion. Once aspirated, mosquitoes were counted and then transferred to a small plastic cup covered in bridal veil. Approximately 10 females and 10 males were used for each concentration. Each cup of mosquitoes were then sprayed with two pumps of a 50 ml fingertip sprayer (The Bottle Crew, West Bloomfield, MI) containing the methoprene test solution. Droplet size produced by these sprayers was  $0.17 \pm 0.04$  mm, droplet density  $2.5 \pm 0.7$  droplets per  $\text{mm}^2$ , determined through the use of water sensitive paper (Teejet, Springfield, IL). During application the sprayer was held 10 cm away from the container. After application of the solution, mosquitoes were moved to cages similar to those described in Chapter 1. Each cage of mosquitoes was provided a sucrose soaked wick to feed on ad libitum.

Three days after dosing, mosquitoes were provided one anesthetized mouse (IACUC #00905A2005) to feed on for 15 min. After feeding, one oviposition cup constructed of a piece of seed germination paper placed in a plastic 50 ml cup half full of water was set in each cage. After 1 wk (allowing for the completion of one gonotrophic cycle) the oviposition cup was replaced and the mosquitoes were allowed to bloodfeed again. At this time mortality and numbers of eggs were recorded. This process was

completed for two full gonotrophic cycles (14 d). Each paper was dried for one week before being submerged in 500 ml water with 1ml 6.0% liver powder solution for 24 hours. After this period the numbers of larvae present were counted. This experiment was replicated five times for each species.

## **Treatments**

### *Aedes aegypti*

Mosquitoes were treated with 10% Altosid® diluted in water and 1% methoprene diluted in acetone. The 10% Altosid® solution equates to a 0.5% methoprene application, half as concentrated as the highest methoprene concentration used in prior experiments (Chapters 2 and 3). Control solutions were water and acetone control solutions. The inclusion of a methoprene treatment provided a baseline of comparison between this experiment and earlier work (Chapter 2).

### *Aedes polynesiensis*

Mosquitoes were treated with 10%, 1.0%, and 0.1% Altosid® solutions, plus a water control. These treatments equate to 0.5%, 0.05%, and 0.005% of the active ingredient, methoprene. *Ae. polynesiensis* has been shown to be less capable of withstanding methoprene treatment (Chapter 3), therefore a range of treatments was used similar to previous work.

### *Aedes albopictus*

Mosquitoes were treated with 10% Altosid® and water control solutions, in order to assess the effects of commercial grade methoprene on wild type mosquitoes.



Concentrations of Altosid® were made by diluting commercial grade Altosid® or 99.97% technical grade methoprene (Central Life Sciences, Dallas, TX) with water or acetone, respectively. Altosid® is 5% methoprene by volume. The control was water alone.

## **Statistics**

Mortality of males and females was assessed using a Cox Proportional Hazards Analysis to examine for significant differences among treatments. Comparison between species was calculated by first correcting mortality using Henderson-Tilton's calculation (Henderson and Tilton 1955), performing arc sine square root transformation on the resulting proportions, and then performing an analysis of variance. Kruskal-Wallis nonparametric analysis of variance was used to examine for significant differences in fecundity among experimental treatments ( $\alpha = 0.05$ ) for fecundity measures (eggs per female and number of larvae). Pairwise comparisons were made using the Dunn method. All analyses were performed in JMP v.9 (SAS Institute Inc., Cary, NC).

## **Results**

### *Aedes aegypti*

Spray application of methoprene has a significant effect on female mortality ( $\chi^2 = 37.4$ ;  $P < 0.0001$ ; Table 4.1). Female mosquitoes treated with 1.0% methoprene spray experienced significantly higher mortality than control or 10% Altosid® treatment sprays ( $P < 0.0001$ ). Altosid® treatment caused significantly higher mortality than acetone control ( $P = 0.001$ ), but not the water control ( $P = 0.3$ ). Male mosquitoes also

experienced a significant difference in mortality ( $\chi^2 = 10.0$ ;  $P = 0.02$ ); those treated with 1.0% methoprene spray solution experienced higher mortality than both water and acetone controls ( $P = 0.005$ ), and 10% Altosid® treatment ( $P = 0.02$ ). There were no significant differences between the controls.

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2 = 0.01$ ;  $P = 0.93$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a significant effect on fecundity ( $\chi^2 = 18.5$ ;  $P < 0.001$ ). Females treated with the acetone control spray laid significantly more eggs than both the 1.0% methoprene ( $P = 0.002$ ) and the 10% Altosid® treatment ( $P < 0.001$ ). Females treated with the water control spray laid significantly more eggs than those treated with the 1.0% methoprene solution ( $P = 0.01$ ) and the 10% Altosid® treatment ( $P = 0.004$ ). The methoprene spray had a significant effect on the number of neonate larvae produced by each treated female mosquito ( $\chi^2 = 17.1$ ;  $P < 0.001$ ). Acetone control females had significantly more offspring resulting from their eggs than both 1.0% methoprene ( $P < 0.001$ ) and 10% Altosid® ( $P = 0.0001$ ) treatments. The water control treated females produced significantly more eggs than both the 1.0% methoprene ( $P = 0.03$ ) and 10% Altosid® ( $P < 0.01$ ) solutions (Table 4.1).

#### *Aedes polynesiensis*

Spray application of Altosid® has a significant effect on female mortality ( $\chi^2 = 8.4$ ;  $P = 0.04$ ). Female mosquitoes treated with 10% Altosid® spray experienced significantly higher mortality than control ( $P = 0.02$ ), 0.1% Altosid® ( $P = 0.01$ ), and 1.0%

Altosid® ( $P = 0.02$ ) treatment sprays. There were no significant differences between the control and the lower treatment levels. Male mosquito mortality was not significantly affected by Altosid® application ( $\chi^2 = 2.0$ ;  $P = 0.5$ ; Table 4.2).

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2 = 0.0009$ ;  $P = 1.0$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a significant effect on fecundity ( $\chi^2 = 16.6$ ;  $P < 0.001$ ). Females treated with the water control spray laid significantly more eggs than the 0.1% Altosid® treatment ( $P = 0.05$ ), 1.0% Altosid® ( $P < 0.0001$ ), and 10% Altosid® ( $P < 0.0001$ ) treated mosquitoes. Females treated with 0.1% Altosid® laid significantly more eggs than those treated with the 1.0% Altosid® solution ( $P = 0.005$ ) and the 10% Altosid® treatment ( $P = 0.004$ ). The methoprene spray had a significant effect on the number of neonate larvae produced by each treated female mosquito ( $\chi^2 = 18.0$ ;  $P < 0.001$ ). Water control females had significantly more offspring resulting from their eggs than both 1.0% Altosid® ( $P = 0.002$ ) and 10% Altosid® ( $P = 0.002$ ) treatments (Table 4.2).

### *Aedes albopictus*

Spray application of Altosid® did not significantly affect female mortality ( $\chi^2 = 0.1$ ;  $P = 0.7$ ). Male mosquitoes also did not experience significant mortality from Altosid® treatment ( $\chi^2 = 0.09$ ;  $P = 0.8$ ; Table 4.3).

There were no significant differences in fecundity values between the two observed gonotrophic cycles ( $\chi^2 = 0.01$ ;  $P = 0.92$ ), therefore fecundity values for both cycles were consolidated for analysis. Treatment with a methoprene spray had a

significant effect on fecundity ( $\chi^2 = 6.4$ ;  $P = 0.01$ ). Females treated with the water control spray laid significantly more eggs than the 10% Altosid® treatment ( $P = 0.01$ ). The methoprene spray had a significant effect on the number of neonate larvae produced by each treated female mosquito ( $\chi^2 = 9.3$ ;  $P = 0.002$ ). Acetone control females had significantly more offspring resulting from their eggs than those treated with 10% Altosid® ( $P = 0.003$ ; Table 4.3).

#### Comparison of species

There were no significant differences in mortality found between the three species observed in this study for males ( $P = 0.63$ ; Figure 4.1B) or females ( $P = 0.75$ ; Figure 4.1A).

### Discussion

The results of this study indicate that commercial grade methoprene is capable of reducing the fecundity of adult female mosquitoes, similar to prior work with technical grade methoprene. Mosquitoes experienced an acute toxic effect from high levels of methoprene dosage, causing significant mortality within 3 d post bloodmeal (Chapter 2). When treated with Altosid®, there was a less dramatic effect on mosquito mortality than with technical grade methoprene, shown by the higher number of mosquitoes capable of surviving until the second gonotrophic cycle (17 d) than previously noted (Chapter 3).

Female *Ae. aegypti* mosquitoes experienced significantly higher mortality when sprayed with water than acetone control solutions. Mosquitoes treated with water are prevented from flying for a period after treatment, until their wings become dry (Personal

observations). The differences found could have been due to the time it takes the water to evaporate from the wings of the mosquitoes being longer than acetone.

*Ae. polynesiensis* and *Ae. albopictus* males were not significantly affected by the application of Altosid®, indicating that Altosid® treatment has a less severe effect on the males than technical grade methoprene. Female mosquitoes still suffer from acute mortality, though this mortality was not as severe as previously noted (Chapter 2 and 3).

The differences between species in both fecundity and mortality measures found in this study may have been due to the varying role of JH in treated mosquitoes. In female mosquitoes, JH determines the early development of the mosquito ovaries during vitellogenesis (Raikhel 2002). Vitellogenesis immediately precedes the resting stage of the mosquito ovaries, where the ovaries halt at a length of 100 µm until the mosquito takes a bloodmeal (Noriega 2004). Once bloodfed, JH levels decline, and ecdysone levels increase (Dhadialla and Raikhel 1994). Ecdysone increase initiates the development of the mosquito ovarioles into eggs. Results from prior experiments (Chapter 2) indicate that methoprene treatment in adult female *Ae. aegypti* mosquitoes causes a suspension of ovariole development, where ovarioles become halted at the resting stage. *Ae. polynesiensis* and *Ae. albopictus* mosquitoes experience a different reaction to methoprene treatment, where the chorion of the developing eggs become weaker and leak yolk on dissection. While the mosquitoes which experienced this reaction were capable of oviposition, the eggs that were laid were unable to hatch. Further research could illustrate if this is due to a loss of nutrition from yolk lost during egg development. There may be a connection between the treatment reaction of each mosquito with the difference between these species in length of development time.

Because *Ae. polynesiensis* and *Ae. albopictus* have longer development times (personal observations), it is possible that they are more capable of compensating for the presence of methoprene in the haemolymph.

When corrected using the Henderson-Tilton formula, there were no significant relationships found in mortality between each of the mosquito species studied. This relationship is true for both males and females. The absence of significant mortality effects is beneficial to the strategy of Altosid® treatments with incompatible releases and indicates that treated males will be able to survive sufficient periods to assist the release program.

Increased survival in addition to lowered fecundity indicates the viability of an Altosid® treatment to assist *Wolbachia* SIT. Before male mosquitoes may be released into the environment, they must first be sorted from the females. Current sorting methods are not perfect, and it is possible for female mosquitoes to be missed during the separation process (Mains 2012). By applying a 10% diluted Altosid® solution to sorted mosquitoes prior to release, researchers can reduce the potential for fertile females to be released into the environment. Results of this research indicate that any male mosquitoes exposed to an Altosid® treatment will be capable of maintaining competitive status with wild males. Further research should be performed to ascertain the exact physiological response of male *Aedes* mosquitoes to methoprene treatment. Research has not been performed to determine the precise role JH has in spermatogenesis, and further research could show any chronic effects on male virility, similar to the effects on female mosquitoes. Other studies have already indicated that male *Wolbachia* infected mosquitoes are capable of equivalent levels of competition to wild males (Chambers *et al*

2011), and the results of this study indicate that Altosid® treatment has no acute detrimental effect on male health and virility.

By using Altosid®, scientists hoping to control mosquitoes using SIT have a simple method to deactivate potentially released females. The method currently described by this study uses a diluted form of currently available commercial grade materials, and is therefore accessible and not cost prohibitive. Low cost and ease of use are important for SIT releases, due to the large numbers of insects required to establish control. In the case of the *Wolbachia* biopesticide method, repeated, inundative releases of mosquitoes are required in order to establish population control (Alphey *et al* 2010). By using Ultra Low Volume (ULV) sprays, small amounts of Altosid® could potentially be used to treat containers of mosquitoes prior to release. Future studies should ascertain the optimum volume container and droplet size for methoprene application to potentially massive amounts of mosquitoes. There is a possibility that mosquitoes which were still capable of ovipositing during the course of this project were simply missed by the spray method used. The use of a ULV spray corrects this issue by shrinking the size of the droplets and increasing the number of droplets introduced to the atmosphere (Burt and Smith 1974). The results described here support additional research in this area.

**Table 4.1:** *Ae. aegypti* mortality and fecundity measures during Altosid® application experiments

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
3 Days	Acetone	0.0±0.0	8.5±0.3	---	---
	Water	0.0±0.0	0.0±0.0	---	---
	10% Altosid	3.4±4.8	3.3±4.6	---	---
	1% Methoprene	43.6±23.1	28.6±14.6	---	---
10 Days	Acetone	3.9±5.4	46.9±7.4	---	---
	Water	24.9±17.2	42.9±8.4	---	---
	10% Altosid	24.7±11.4	50.5±27.4	---	---
	1% Methoprene	71.8±11.6	70.5±28.9	---	---
17 Days	Acetone	11.5±5.4	57.6±10.8	48.9±37.5	43.7±35.1
	Water	39.6±18.0	62.5±17.3	33.8±25.9	23.5±14.7
	10% Altosid	49.8±20.6	76.7±26.6	0.6±1.8	0.0±0.0
	1% Methoprene	87.6±8.1	100.0±0.0	0.0±0.0	0.0±0.0

**Table 4.2:** *Ae. polynesiensis* mortality and fecundity measures during Altosid® application experiments

Time	Treatment	% Female mortality	% Male mortality	No. of eggs per female	No. of larvae per female
3 Days	Control	10.0±17.3	36.1±24.1	---	---
	0.1% Altosid	13.3±15.3	36.7±5.8	---	---
	1.0% Altosid	27.6±23.7	46.7±15.3	---	---
	10% Altosid	16.7±5.8	38.8±10.2	---	---
10 Days	Control	43.2±24.2	81.7±16.1	---	---
	0.1% Altosid	46.7±5.8	93.3±5.8	---	---
	1.0% Altosid	68.5±11.7	96.7±5.8	---	---
	10% Altosid	60.0±20.0	70.9±10.1	---	---
17 Days	Control	54.6±22.2	97.2±4.8	26.1±13.2	17.0±15.2
	0.1% Altosid	76.7±5.8	96.7±5.8	15.8±9.9	8.4±5.4
	1.0% Altosid	77.6±15.9	100.0±0.0	0.5±0.9	0.0±0.0
	10% Altosid	86.7±5.8	100.0±0.0	0.0±0.0	0.0±0.0



**Table 4.3:** *Ae. albopictus* mortality and fecundity measures during Altosid® application experiments

Time	Treatment	% Female Mortality	% Male Mortality	No. of eggs per female	No. of larvae per female
3 Days	Control	23.3±32.5	26.7±17.2	---	---
	10% Altosid	9.8±10.0	33.0±30.8	---	---
10 Days	Control	33.3±34.3	69.4±23.4	---	---
	10% Altosid	42.4±26.1	78.2±26.9	---	---
17 Days	Control	74.4±28.0	96.3±4.5	30.9 ±8.6	13.7±8.1
	10% Altosid	65.1±20.5	100.0±0.0	14.8±12.3	2.1±2.3

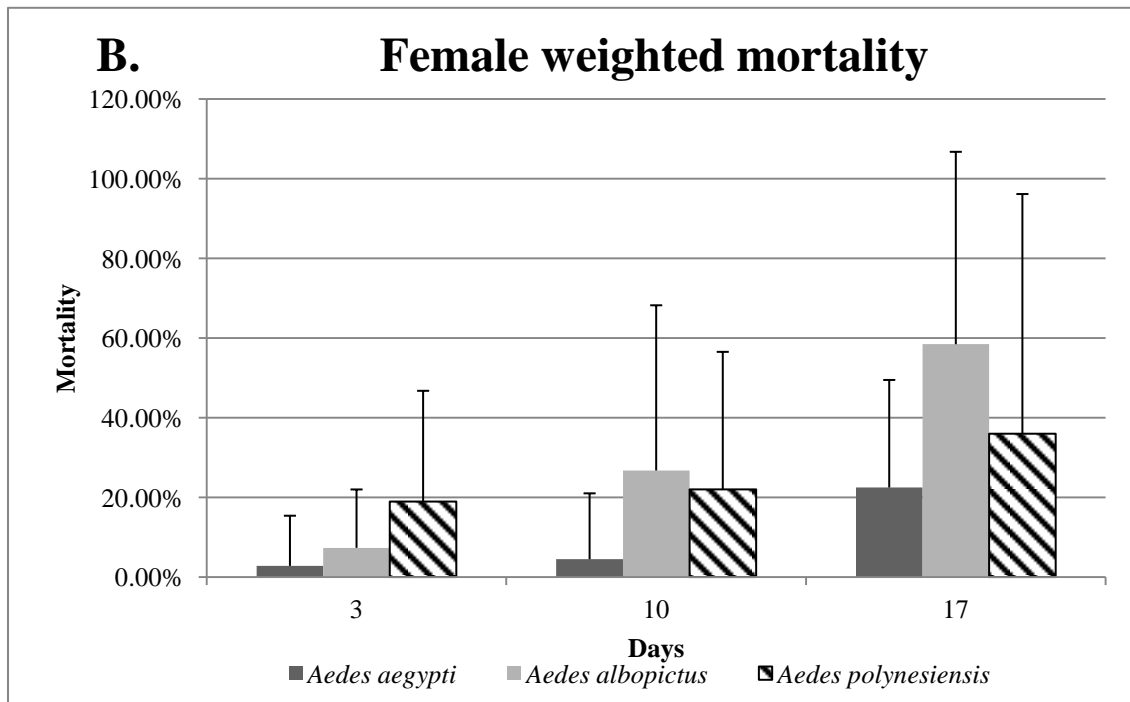
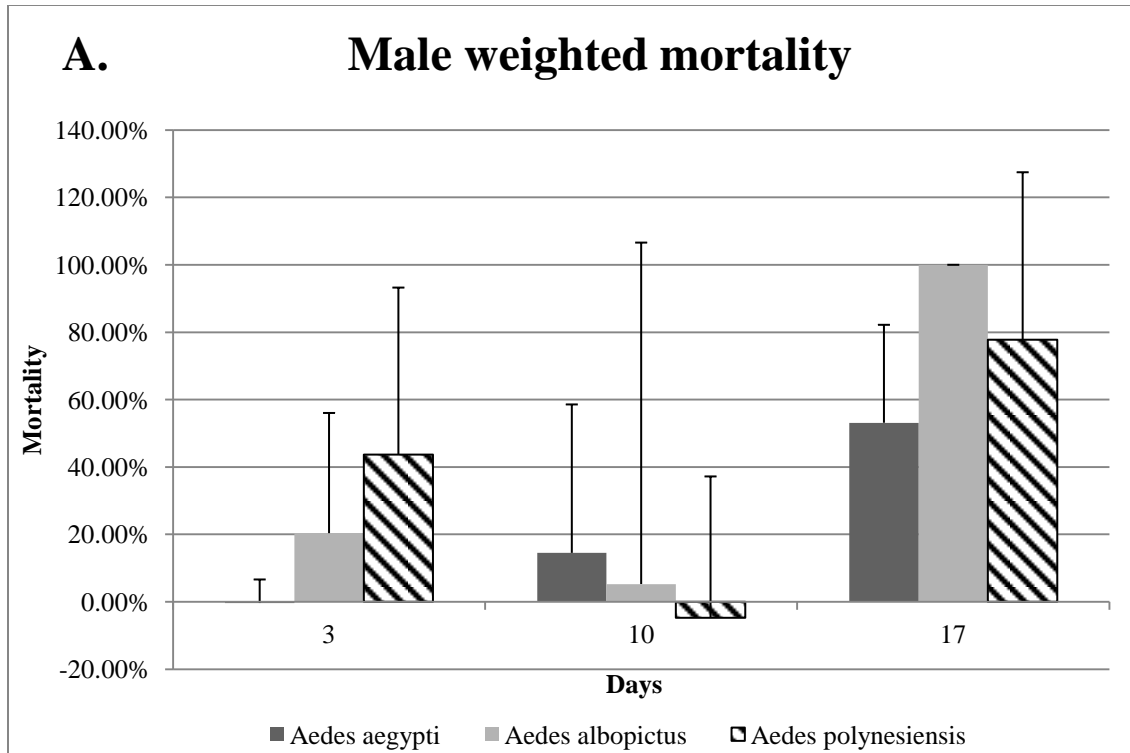


Figure 4.1: Weighted mortality of Altosid® treated mosquitoes, by sex. These numbers have been corrected for treatment levels using the Henderson-Tilton equation. Bars show standard deviation in mortality between replicates (N = 5). A) Male mortality B) Female mortality

## Chapter Five

### Conclusion

Mosquito population control is a vast and daunting goal facing society. Methoprene application has the potential to enhance current control measures, and perhaps contribute towards long term control of mosquito populations. The experiments shown in prior chapters illustrate the potential of methoprene to have chronic effects upon female mosquito reproductive capacity. These chronic effects could be used to establish long term mosquito control by supplementing incompatible male release or through direct application through aerosol treatment.

Incompatible male release functions by releasing male mosquitoes artificially infected with a symbiont, *Wolbachia*. These males are incapable of successfully producing offspring with any female mosquito that is not infected with the same strain of *Wolbachia* (Werren 1997). By inundating the wild population with infected males, the wild females are effectively prevented from successfully breeding, and the population is reduced (Dobson 2003). Before release, all female mosquitoes must be removed to prevent replacement of the wild population with *Wolbachia* infected females. The current sorting methods use a glass plate to physically separate male pupae from female pupae (Mains 2012). This method is not perfectly effective, and some females can be overlooked. Current measures to prevent this error involve the anesthetization of all the mosquitoes prior to release, and physically removing any females. This method is time consuming, and some females could still be overlooked (personal observations). Methoprene application prior to release could greatly simplify this process. The results of earlier chapters indicate that a spray composed of 0.1% methoprene would

successfully arrest the reproductive development of any missed female mosquitoes, while not having any significant effects upon male mortality.

Methoprene application would significantly reduce the amount of processing needed prior to incompatible male release. Adult mosquitoes could be isolated in cages within one room, where a mister system would then treat all cages containing mosquitoes at the same time. Any female mosquitoes contained within would have their reproductive capacity significantly reduced, which in turn significantly reduces the chances of population replacement. Also, since methoprene does not have a significant effect upon male fitness, methoprene treated incompatible males will still be capable of mating with wild females, and will still be capable of effecting wild mosquito population levels.

In pest control situations, methoprene could function as an air dispersed chronic insecticide. By applying methoprene within an Ultra-Low Volume (ULV) spray, pest control technicians could easily treat adult mosquitoes which could not have been easily reached using prior methods. This is particularly important when attempting to control *Aedes* mosquitoes, which breed using small cryptic water sources that are difficult for a technician to find (Benedict *et al.* 2007). There is also a potential for the spray to serve the purpose of treating the small water sources where *Aedes* mosquito larvae live, and preventing those larvae from becoming adults. By simultaneously affecting adult fecundity and larval survival, methoprene application could have a significant effect on mosquito population size.

Methoprene is currently commercially available as the active ingredient within the liquid larvicide Altosid®, and contains 0.5% methoprene by volume. Altosid® is a

pesticide commonly applied by mosquito control agencies, and is inexpensive to acquire in comparison to technical grade methoprene. A 10% Altosid® dilution resulted in 0.05% methoprene application to adult female mosquitoes, and was shown to effectively reduce female mosquito reproduction when applied by spray in prior chapters.

Liquid Altosid® is currently applied as an aerial spray, but is still for the express purpose of treating water sources. When Altosid® is applied by aerial spray, it is for the treatment of fields which have become flooded and mosquito populations have increased to the point that civilians have begun to complain. Aquatic non-target effects have been researched for methoprene, but little research has been performed on terrestrial insect species due to the constraints of methoprene application. The aerial treatment method is common in crops that use flood irrigation, like rice and dates. In these cases, non-target terrestrial effects are not evaluated, since there are no beneficial insects which are considered in these situations.

This work could be effectively expanded upon by applying an ULV spray to a number of insects which naturally occur in settings where the pesticide could be applied, in order to ascertain non-target effects of methoprene application. This could be further narrowed to focus on beneficial urban insects, since methoprene treatment would be used primarily in urban areas. Mosquitoes are difficult to control in urban areas, and small water sources are common. Methoprene is already used in urban environments as a larvicide to treat man-made bodies of waters which have become infested with mosquitoes. With successful testing, aerial Altosid® application could supplement urban control strategies, and greatly decrease mosquito populations in urban environments.

ULV application could treat any adults within the treatment area, and prevent female mosquitoes from ovipositing outside the treatment area.

Methoprene application has the potential to assist with multiple strategies of mosquito control, both through directly effecting the pest population and by working in conjunction with other control methods. Methoprene is already a part of mosquito integrated pest control; however it has the potential to become a much larger part through chronic effects. The long lasting effects discussed in prior chapters, where fecundity is significantly reduced for multiple gonotrophic cycles, could contribute to more complete control over mosquito populations. Further research is needed before a new methoprene application regimen can be implemented, most notably looking at the non-target effects of methoprene application in field situations.

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