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The Manipulation and Examination of *Wolbachia* in Medically Important Mosquitoes

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The Manipulation and Examination of *Wolbachia* in Medically Important Mosquitoes

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Masters of Science in the
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By

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ABSTRACT OF THESIS

The Manipulation and Examination of
Wolbachia in Medically Important Mosquitoes

Mosquitoes are a major vector of human disease and result in massive costs to public health in affected regions. It has been suggested that *Wolbachia pipientis* could be used for mosquito population reduction. *Wolbachia* is a maternally-transmitted endosymbiont of arthropods and nematodes that infects the cytoplasm of host cells. In mosquitoes, *Wolbachia* manipulates reproduction through Cytoplasmic Incompatibility (CI), which is characterized by a cessation of embryonic development in certain crossing-types. However, the relationship between *Wolbachia* and its host is complex and not fully understood. The crossing relationships between naturally-infected and aposymbiotic populations of *Culex pipiens pipiens* and *Culex pipiens molestus* were examined in order to better understand the effects of CI on life history traits such as egg production and egg hatch. Hatch consistent with a unidirectional incompatibility relationship was observed. However, low egg production was also observed in some crossing-types, implying that *Wolbachia* may manipulate its host in unknown ways. In addition, uninfected mosquito eggs were injected with cytoplasm from infected eggs to generate artificially infected *Culex* lines. While no transinfected lines were successfully generated, several observations were made that may prove useful in future microinjection research.

KEYWORDS: Cytoplasmic Incompatibility, *Wolbachia pipientis*, Cytoplasmic Microinjection, *Culex*, Egg Production

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The Manipulation and Examination of
Wolbachia in Medically Important Mosquitoes

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The successful completion of my studies would not have been possible without the constant support of my parents, friends, and partner. This Thesis is dedicated to them.

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Introduction

Impact of Mosquitoes on Human Health

Mosquitoes are the most significant animal vector of human pathogens. According to the World Health Organization, over half of the world population is at risk for diseases such as malaria and yellow fever (World Health Organization, 2014).

Beyond the cost in human lives, mosquito-borne disease inflicts a significant economic cost resulting from losses in labor, productivity, and lifespan. This damage is quantified using Disability-adjusted Life Years (DALY), which measures the average years of productivity lost due to disease within a population. According to the World Health Organization, in 2012 the worldwide toll of malaria represented a loss of 778.9 years per 100,000 people. Considering the extreme cost associated with mosquito-borne pathogens, it is unsurprising that a great deal of attention is given to finding avenues to reduce occurrence.

Historically, a primary method of mosquito control has been the widespread use of chemical insecticides. Initiatives such as the National Malaria Eradication Program, which occurred from 1947-1951 in the United States, succeeded in eliminating local malaria transmission from the American Southeast through widespread insecticide application. However, similar initiatives such as the World Health Organization's Global Malaria Eradication Programme failed to produce lasting results in larger geographic regions (Waldemar, 2009; Sledge and Mohler, 2013). As such, a great deal of effort has been exerted into developing alternative methods of mosquito control. These have been quite varied, from the production of genetically modified mosquitoes to radiation-sterilized mosquitoes, but each has presented its own challenges (Knols et al., 2007; Klassen, 2009). One such avenue, which has received

substantial interest and attention, has been the α-proteobacterial endosymbiont *Wolbachia pipientis*.

Classification and Distribution of *Wolbachia*

Originally described as a rickettsia-like bacterium in *Culex pipiens*, it is now known that *Wolbachia pipientis* is a monophyletic group of endosymbiotic bacterial strains that is closely related to other *α-proteobacteria* such as *Neorickettsia*, *Anaplasma*, and *Ehrlichia* (Hertig and Wolbach, 1924; Hertig, 1936; O'Neill et al., 1992). Within *Wolbachia*, these bacterial strains are clustered into multiple clades based on genetic similarity. Each clade tends to be distributed within an individual host group, though multiple clades can exist within and even co-infect certain groups (Werren et al., 1995; Lo et al., 2007). For example, the Genus: *Aedes* is host to both the A and B clades; and in the case of *Aedes albopictus* this takes the form of a superinfection, where both A and B *Wolbachia* infect the same organism (Sinkins et al., 1995). This differs from *Culex* mosquitoes, which appear to only carry the B supergroup (Zhou et al., 1998). Historically, it was believed that *Anopheline* mosquitoes lacked a native *Wolbachia* infection. Recent research has shown evidence for a B supergroup infection in some populations of *Anopheles gambiae* (Baldini et al., 2014).

Wolbachia distribution is not limited to mosquitoes, but is common within other arthropods and nematodes, being present in as much as 40% of terrestrial arthropods (Zug and Hammerstein, 2012). Within infected species, the frequency of infection in localized populations can approach fixation. This ubiquity is often attributed to the ability of *Wolbachia* to establish itself within host populations through reproductive manipulations (Engelstadter and Hurst, 2009).

Wolbachia in Populations

Wolbachia has evolved a number of intriguing mechanisms that can increase its frequency within a host population such as male-killing, feminization of genetic males, induction of thelytokous parthenogenesis, and Cytoplasmic Incompatibility (CI). Each mechanism enhances the proportion of infected females in order to drive the frequency of infection towards fixation.

Three of these, namely male-killing, feminization, and parthenogenesis, alter the sex ratio to increase the relative population of *Wolbachia* infected females (Cordaux et al., 2011). In male-killing, *Wolbachia* kills a large percentage of infected males in each clutch. This imparts an advantage in infected female siblings through reduced resource competition and crowding during development (Zug and Hammerstein, 2014).

In cases of parthenogenesis-inducing *Wolbachia*, infected females lay unfertilized diploid eggs that develop into females. Since infection is vertically transmitted through the egg, this mechanism increases the proportion of the population capable of transmitting the infection (Cordaux et al., 2011; Werren 2011). Similarly, feminzation-inducing *Wolbachia* increases the proportion of infected females by causing genetic males to develop as females (Kageyama et al., 2002).

In contrast, CI biases infection by reducing egg hatch in uninfected females who mate with infected males (Cordaux et al., 2011). Incompatible crosses result in the cessation of early embryonic development. This can be described using a Modify/Rescue model, where the sperm in infected males is modified to induce mortality if not exposed to a factor in the egg that rescues normal development (Poinsot et al. 2003, Engelstädter and Hurst 2009).

Cytoplasmic Incompatibility Relationships between *Wolbachia* Strains

There are cases where two *Wolbachia* strains interact during reproduction. In such cases CI relationships can be further complicated. One potential outcome is known as Unidirectional Incompatibility, where low hatch is observed along one direction of a cross but not in the reciprocal cross. A second potential outcome is a situation known as Bidirectional Incompatibility, where the interaction between two strains of *Wolbachia* results in incompatibility in reciprocal crossing directions. With both uni- and bi-directional CI, incompatibility can be partial, resulting in a mix of viable and inviable eggs, or complete, in which very few remain viable (Engelstädter and Hurst 2009).

Both unidirectional and bidirectional incompatibility have been observed between different strains of native *Cx. pipiens Wolbachia* (*w*Pip), even between naturally occurring populations in geographically small regions. Additionally, CI relationships have been shown to change in colony reared systems in as little as 50 generations (Engelstädter and Hurst, 2009; Duron et al., 2012; Bourtzis et al., 2014). This diversity and susceptibility to change makes understanding the fundamental relationship between *Wolbachia* and its *Culex* host difficult. Theoretically, one could examine the host's part in this relationship by transinfecting a *Culex* line with a foreign strain of *Wolbachia* such as *w*AlbB, the B clade *Wolbachia* naturally infecting *Aedes albopictus*.

***Wolbachia* as a Reproductive Parasite**

Regardless of the method *Wolbachia* utilizes to bias reproduction, it is capable of rapidly driving itself into populations, even if the infection incurs a decrease in host fitness. In both CI and parthenogenesis-inducing *Wolbachia*, infection has been found to be linked to reduced

lifespan and fecundity in infected females (Hoffmann et al., 1990**;** Stouthamer and Luck, 1993). Similarly, male-killing *Wolbachia* can spread to high prevalence despite imposing a high fitness cost to infected females, severely reducing offspring survivorship (Jiggins et al., 2002). This has led to *Wolbachia* being considered a reproductive parasite.

But, in recent years there have been multiple instances of *Wolbachia* imparting some fitness benefit to its host (Giordano et al., 1995; Hoffmann et al., 1996; Perrot-Minnot et al., 2002; Bian et al., 2010). In many such cases *Wolbachia* can simultaneously benefit its host and acts as a reproductive parasite, resulting in a "Jekyll and Hyde" infection (Jiggins & Hurst, 2011). Selection on CI-inducing *Wolbachia* favors strains that increase relative fecundity in infected females, even if this reduces the strength of CI (Turelli, 1994). Such an increase in fecundity would provide a competitive advantage in situations where two incompatible *Wolbachia* strains occur within the same geographic range, as it would help to offset a population reduction resulting from CI.

In addition to benefits conferred through natural infection, unexpected advantageous traits have been observed in artificial infections. Most notably among these is the observation that some strains of *Wolbachia* can impart pathogen resistance in mosquito hosts (Teixeira et al., 2008; Moreira et al. 2009; Hughes et al. 2011; Bian et al., 2013; Caragata and Moreira 2016). Given that several RNA viral pathogens of humans, such as Dengue, are vectored by mosquitoes, this observation has sparked exploration into the use of *Wolbachia* based viral resistance as a means of disease control (Bian et al., 2010).

Wolbachia Mediated Inundative Release Programs

Early work in CI was conducted before *Wolbachia* had been demonstrated as the causative agent (Laven, 1969). This work was facilitated through the translocation of existing *Wolbachia* infected populations to new geographic regions. In contrast, the majority of current release programs focus on artificially infected mosquito lines. Because the generation of artificial lines allows researchers to choose non-native *Wolbachia* strains that generate bi-directional CI, decreasing the probability that the accidental release of females could result in the establishment of compatible populations and rendering further releases ineffective.

Artificial lines in mosquitoes are generated by injecting cytoplasm that contains *Wolbachia* from a naturally infected host and into a newly laid egg of an uninfected organism. As the egg goes through cellularization, the injected *Wolbachia* becomes incorporated into the tissue of the host, resulting in an individual infected with and capable of transmitting the infection to its offspring. The recipient may be either uninfected lines or those already carrying a different infection. Historically these transinfected lines are largely limited to generated *Aedes* mosquito lines, although recently a wAlbB infection was induced in *Anopheles stephensi* (Xi et al., 2005; Bian et al., 2013). However, as of yet there have been no successfully transinfected *Culex* mosquito lines.

Wolbachia-transinfected lines can theoretically be utilized in two ways. One way is to introduce large numbers of artificially infected male and female mosquitoes into a population in order to outcompete the existing population and establish the new infection in the release area. This is known as Population Replacement, and it relies heavily on observed partial RNA-viral resistance in generated *Aedes* lines (Hoffmann et al., 2011; Wong et al., 2011; Rances et al., 2012). However, the long-term efficacy of such programs has been questioned based on the

possibility that viral resistant phenotypes are caused by upregulation of antiviral pathways resulting from the new infection (Zug et al., 2015). This is further corroborated by observations that antiviral effects are rare in naturally occurring infections (Vavre and Charlat, 2012).

An alternate way to utilize *Wolbachia*-transinfected lines is through the release of male mosquitoes into wild populations. These males would compete with wild males, obtaining some portion of the mating events, and reducing the wild population through CI (Bourtzis et al., 2014). However, given *Wolbachia's* tendency to rapidly drive itself into a population, the accidental release of females could quickly render the program ineffective. This risk is mitigated somewhat through the utilization of crosses that induce bidirectional incompatibility, because any accidentally released females would be unable to reproduce when they mate with incompatible wild type males, thus preventing the establishment of the novel *Wolbachia* infection in the breeding population (Dobson et al., 2002).

Research Intent

The relationship between *Wolbachia* and its *Culex* host is poorly understood. Attempts to better understand this relationship are confounded by the varied and fluid nature of CI relationships within *w*Pip and the lack of transinfected *Culex* lines. Additionally, the establishment of a transinfected *Culex* mosquito line could aid the development of mosquito control programs. Taking this into account, I designed experiments to accomplish three tasks. First, to examine two life history traits (egg production and hatch) of naturally-infected and aposymbiotic *Culex pipiens pipiens* and *Culex pipiens molestus*. Second, to examine the CI relationships and short-term effects on these life history traits in cases of hybridization. Third, to examine the effects of foreign *Wolbachia* introduction on life history through the establishment

of artificially generated *Cx. p. pipiens* and *Cx. p. molestus* lines. Life history traits and CI relationship analysis were measured over multiple 5x5 crossing experiments. Finally, the development of novel transinfected lines would be achieved through the modification of existing cytoplasmic microinjection techniques.

While the establishment of aposymbiotic lines and CI relationship analysis were accomplished successfully, I was unable to to produce a transinfected *Culex* mosquito line. However, analysis of my methodology may prove useful to others attempting the same process.

The Removal of *Wolbachia* from Two Medically Important Mosquitoes and Examining for an Effect on Reproduction

Introduction:

Wolbachia pipientis is an α-proteobacterial endosymbiont infecting the cytoplasm of host cells. Infection is vertically transmitted and can bias infection rates through processes such as male killing, feminization of genetic males, parthenogenesis, and early embryonic death (Sinkins 2004). The latter these, known as Cytoplasmic Incompatibility (CI), can occur when a *Wolbachia* infected male mates with either an uninfected female or with a female that is infected with an incompatible *Wolbachia* strain. Cytoplasmic Incompatibility has been described using a Modify/Rescue model, where the sperm is modified and induces mortality if not exposed to a factor in the egg that rescues normal development (Poinsot et al. 2003, Engelstädter and Hurst 2009).

Within the *Culex pipiens* complex, CI-Host interactions have been observed to be unusually complicated. Comparisons of *ank*2 and *pk*1 genes show the presence at least 100 genetically distinct *w*Pip strains belonging to five sub-clades spread throughout complex (Atyame et al., 2011; Dumas et al., 2013). Variations within the *Culex pipiens* complex have been shown to occur both between and within these sub-clades (Barr, 1980; Magnin et al., 1987; Duron et al. 2012). Functionally, this means that closely related subspecies within the complex can experience differing levels of incompatibility (Duron et al., 2006; Atyame et al., 2014). Variation in CI has even been observed between members of the same species within geographically contiguous regions (Duron et al., 2012).

Research focusing on how *Wolbachia* strains interact with each other and *Aedes* mosquitoes has been extensive, comparatively little attention has been given to how *Wolbachia* affects its *Culex* hosts (Sinkins et al., 2005; Almeida et al., 2011). Such examinations of *Culex*-*Wolbachia* relationships may help to parse the complicated interactions within this species complex.

With this in mind, I compared several life history traits in naturally-infected and aposymbiotic lines of two closely related subspecies that regularly inhabit similar environments, the obligate blood-feeding (anautogenous) *Cx. p. pipiens* and the facultative blood-feeding (autogenous) *Cx. p. molestus* (Kading, 2012). First, I examined if the loss of *Wolbachia* infection affected hatch rate or egg production. Second, I observed for the occurrence of Cytoplasmic Incompatibility in crosses between infected individuals of the two sub-species. Third, I examined if the loss of infection changed the CI relationship or egg production in hybrid crosses.

Materials and Methods:

Mosquito Lines

Two mosquito lines were used in this experiment: A *Cx. p. molestus* "CMM" line carrying a natural *w*Pip *Wolbachia* infection and a naturally-infected *Cx. p. pipiens* "CPP" line (Turrell et al., 2014).

Removal of *Wolbachia* Infection

Removal of *Wolbachia* was conducted using established techniques (Suenaga 1993; Yen and Barr 1973). Approximately 200 mosquito larvae were reared in a hinged lid container with

400ml of distilled water and 2.5ml of bovine liver powder (NOW foods) in solution (60g/L), with larvae exposed to tetracycline (25ppm) from third instar through the remainder of larval development. Adult were placed in a Bioquip 1450 BS collapsible cage and provided 10% sucrose solution. After approximately 7 days, females were provided with a mouse for a blood meal (IACUC protocol # 00905A2005) and a small cup (Conex 163 mL clear portion container) with 0.3mL of liver powder solution (60g/L) and 20 mL dH_2O for oviposition. Three days later, egg rafts were removed from the cage and the rearing process was repeated. After ten generations, treatment with tetracycline was terminated, and the resultant aposymbiotic *Cx. p. pipiens* (CPT) and *Cx. p. molestus* (CMT) lines were reared using normal protocols.

Verification of *Wolbachia* Removal through Polymerase Chain Reaction

At generation 13, infection status was tested via PCR amplification using a *Culex-Wolbachia* specific Orf7c primer set (5'-CCCACATGAGCCAATGACGTCTG-3' forward, 5'-TTGCTTGCTCAACACTTACACTT-3' reverse) (Sanogo and Dobson, 2004). Individuals selected for PCR testing were female adults approximately one-week post-eclosion that had not received a blood meal. DNA was extracted using whole mosquitoes homogenized in 100 µL squash buffer (10 mM Tris – pH 8.2, 1 mM EDTA, 25 mM NaCl) (Gloor et al., 1991). Following extraction, 1 µL of squash buffer homogenate was added 2 µL NEB 10X buffer, 0.5 µL dNTP (10mM), 0.5 µL primers, 0.2 µL NEB Taq, and brought to a total volume of 20 µL using dH_20. This mixture was then amplified in a PTC-200 Thermal Cycler. Samples were denatured at 94˚C for 2 minutes, then cycled 38 times between 94, 55, and 72˚C for 30, 45, and 90 seconds respectively, followed by 72˚C for 10 minutes. Finally, a 7 µL sample of amplified DNA was separated on 1% agarose gel, stained with GelRed Nucleic Acid Gel Stain, and visualized using ultraviolet light.

Crossing of Infected and Uninfected Lines

Five replicates were conducted for each potential cross between CPP, CPT, CMM, and CMT lines. To ensure virginal pairings, pupae were isolated in sealed test tubes and allowed to emerge. Within 24 hours of eclosion, virgin adults were transferred to small cages and provided with sucrose. Each cage contained five females and five males. In cages containing anautogenous females, a blood meal was provided approximately seven days post-emergence. Oviposition sites were then provided approximately three days later. In cages containing autogenous females, no blood meal was given, and an oviposition site was provided approximately ten days post-eclosion. Resulting egg rafts were collected three days after an oviposition site was provided.

Measurement of Crossing Effects

Egg rafts were placed in a petri dish with water. The total number of eggs and number of hatched eggs were counted and recounted 48 and 96-hours after collection. Crosses were grouped together by female cross-type, with naturally-infected intraspecific crosses acting as controls.

Statistical Analysis of Data

Analysis for percent hatch among all compatible crosses was conducted using ANOVA in JMP 10 statistical analysis software. Post-hoc analysis between different crosses was conducted using Tukey-Kramer HSD in JMP 10.

Crossing data were examined using IBM SPSS statistical analysis software. Observed total egg production was found to be non-normal. However, further analysis showed that a square root transformation of total egg production resulted in normality. As a result, further ANOVA analysis of fecundity used the square root of total egg production.

Results:

Compatibility Crossing Results

Egg hatch was observed to fall into two groups. The first group, defined as 'incompatible,' had no observed egg hatch and was made up of the following crosses (female x male type): CMTxCMM, CMTxCPP, CPPxCMM, CPTxCMM, and CPTxCPP crosses. The second 'compatible' group, contained all other cross types. Egg hatch among the compatible crosses was at rates of 88% hatch or higher (Table 2.1).

Egg hatch within the compatible crosses was compared using ANOVA, which showed that differences occurred among groups ($F_{10, 44}$=7.0065, $p<0.0001$). Post-hoc examination of compatible crosses using Tukey-Kramer HSD analysis showed that the hatch rate resulting from one cross was different from all other crosses. Specifically, the egg hatch resulting from the CPPxCMT crosses was lower than other than other compatible crosses (Table 2.1). No hatch occurred among the incompatible crosses, and therefore no statistical analysis of hatch rate could be performed within the incompatible group.

Fecundity Analysis

Variation in egg production was observed among crosses containing infected females. Compared to CPPxCPP crosses, the CPPxCPT crosses were observed to produce significantly more eggs. The interspecific CPPxCMT crosses produced comparable egg numbers to CPPxCPP crosses. However, CPPxCMM crosses produced few eggs (Table 2.2). In contrast, no change in egg number was observed between any crosses involving CMM females, regardless of the male type to which she was mated (Table 2.2).

Variation within egg production was also observed among crosses containing uninfected females. With the exception of one cross, aposymbiotic CPT females were observed to produce comparable egg numbers, regardless of the male mate type. The exception was CPT females mated to CMM males, which produced few eggs. Of the five replicate CPTxCMM cages, four resulted in no eggs (Table 2.2). The remaining cross resulted in 31 eggs, of which none were observed to hatch, as would be expected in an incompatible cross. Aposymbiotic CMT females produced high numbers of eggs, regardless of the male type, even in incompatible crosses (Table 2.2).

Discussion:

In these experiments, I examined the compatibility relationship among infected and uninfected lines of *Cx. p. pipiens* (CPP and CPT respectively) and *Cx. p. molestus* (CMM and CMT). My hypotheses regarding these relationships were two-fold. First, the CI relationship between naturally-infected CPP and CMM would be complete bidirectional incompatibility.

Second, the removal of infection from male-types in incompatible crosses would rescue hatch rate.

Crosses within naturally-infected CPP and CMM strains produced viable eggs. Mating between aposymbiotic CPT individuals resulted in high egg hatch, as did matings between aposymbiotis CMT individuals. As hypothesized, a pattern of complete unidirectional CI in crosses between naturally-infected CPP and aposymbiotic CPT strains was noted. Specifically, high egg hatch was observed in CPPxCPP, CPPxCPT, and CPTxCPT; however, no hatch was observed to result from CPTxCPP crosses. With the CMM and CMT strains, a hatch pattern was observed that was consistent with complete unidirectional CI. Specifically, high egg hatch was observed in CMMxCMM, CMMxCMT, and CMTxCMT, but no hatch was observed in the CMTxCMM crosses.

In examining crosses between naturally-infected and aposymbiotic strains, hybridization of CMTxCPT resulted in high egg hatch, demonstrating there to be no genetic mating isolation that prevents hybridization. A similar result was observed in the CMMxCPT crosses, with high egg hatch resulting. Interestingly, the CMM *Wolbachia* infection was able to rescue the CPP *Wolbachia* type, *i.e*., egg hatch was observed in the CMMxCPP crosses. This is consistent with expectations of CI, because no hatch was observed to result from the CMTxCPP crosses. The hatch pattern suggests that *Wolbachia* plays a role in low observed egg hatch.

A different pattern was observed in the reciprocal crossing direction. No egg hatch was observed in the CPPxCMM crosses, and high egg hatch was observed in the CPPxCMT crosses. The latter suggests that low egg hatch in the CPPxCMM crosses was due to *Wolbachia*-induced CI and not genetic reproductive isolation. The absence of a genetic incompatibility was reinforced by the observation of high egg hatch in the CPTxCMT crosses. Also consistent with

expectations for CI, low egg hatch was observed in the CPTxCMM crosses. However, the latter is complicated by the observed low egg number (discussed below).

The number of eggs produced by CMM and CMT females was relatively consistent, regardless of the male type with which the females were mated. For CPP and CPT females, the resulting egg number was generally consistent, with many eggs produced in all crosses. However the number of eggs produced by the CPPxCPT crosses were significantly higher than all other crosses of CPP or CPT females. The observation of higher egg numbers resulting from crosses with males without *Wolbachia* is unusual and merits further investigation. *Wolbachia* infection has been observed to increase egg production in insect populations (Dobson et al., 2002; Dobson et al., 2004; Weeks et al., 2007). However, the prior examples of increased egg number are associated with the *Wolbachia* infection in females, and there are no examples in which increased egg production results from mating with uninfected males. The number of eggs produced by CPP and CPT females was significantly lower in crosses with *Cx. p. molestus* males. Interestingly, the reduced egg production correlates to the presence of *Wolbachia* in male mates. For both CPP and CPT females mated with CMM males, very few eggs resulted. The low egg number does not appear to result from a genetic factor, but was due to the presence of *Wolbachia* in the male, CPP and CPT females mated with CMT males generated normal numbers of eggs.

Cytoplasmic incompatibility is generally believed to manipulate embryonic development in its host (Bourtzis et al., 2014). However, my observations suggest that the *Wolbachia* infection may affect reproduction between *Cx. p. pipiens* females and *Cx. p. molestus* males. A *Wolbachia*-induced effect on mosquito fecundity has not been described previously. For example, Sinkins et al. (2005) examined CI relationships and egg hatch rates in two strains of *Culex pipiens quinquefasciatus.* While they observed a bidirectional incompatibility relationship

between the two strains, they made no note of a change in egg production. Similarly, Calvitti et al. (2012) examined the CI relationship between *Aedes albopictus* mosquitoes carrying the HTA, HTB, and *w*Pip strains of *Wolbachia*. While they found that the CI relationship can change with male age, they did not note any change in egg production.

Potential explanations for the observed reduction in egg production include a *Wolbachia* induced effect on male mating behavior, e.g., males failing to mate with females. Here, no observations were made of mating behavior or the rate of copulation in crosses. However, in future work this could be observed by replicating this experiment, observing matings, and dissecting females 24 hours after the initial cross to examine for sperm presence in female spermathecae. If the results show that rates of sperm deposit are comparable, an alternative hypothesis is that *Wolbachia* could be modifying egg development through changes in seminal fluid or male accessory gland proteins. Ultimately, additional experiments are necessary to elucidate the full extent to which *Wolbachia* interacts with its hosts in the *Culex pipiens* species complex.

Tables and Figures:

		Male			
		CMM	CMT	CPP	CPT
Female	CMM	95±0.9% N=5	97±0.4% N=5	93±1.4% N=5	94±1.0% N=5
	CMT	0±0% N=5	94±1.1% N=5	0±0% N=5	96±1.2% N=5
	CPP	0±0% N=3	88±1.1%* N=5	94±0.7% N=5	94±0.2% N=5
	CPT	0% N=1	96±0.5% N=5	0±0% N=5	94±0.4% N=5

Table 2.1: Average Percent Hatch of Crossing Types
Grey shaded cells indicate crosses defined as incompatible
Data displayed as Avg ± Std Error. Although five replicate crosses were performed for each cross-type, in some cases, not all crosses produced eggs. Therefore, the number indicates only those crosses resulting in eggs.
* CPPxCMT crosses exhibited a lower hatch rate than all other compatible crosses
Statistical differences were obtained using Tukey-Kramer HSD analysis of One-Way ANOVA comparisons ($p \leq 0.05$)

		Male			
		CMM	CMT	CPP	CPT
Female	CMM	ABC 285±44	ABC 228±53	BC 196±36	BC 192±69
	CMT	AB 361±18	ABC 223±15	BC 194±46	ABC 310±64
	CPP	D 25±12	BC 168±29	C 134±26	A 448±28
	CPT	D 6±6	ABC 259±30	BC 169±22	BC 180±15

Table 2.2: Average Egg Production by Cross
Data displayed as Avg ± Std Error
N=5 for all crosses
Letters signify statistic relationship relative to other crosses
Statistical differences were obtained using Tukey-Kramer HSD analysis of One-Way ANOVA comparisons ($p \leq 0.05$)

Generation of Artificially Infected Mosquito Lines

Introduction:

Wolbachia pipientis is a bacterial endosymbiont infecting in the cytoplasm of many insects and nematodes (Zug and Hammerstein, 2012). In mosquitoes, *Wolbachia* acts as a reproductive parasite and biases increased infection frequency through early embryonic death (Sinkins 2004). This process is known as Cytoplasmic incompatibility (CI), and occurs when a *Wolbachia* infected male mates with an uninfected female or female infected with an incompatible strain.

It has been suggested that CI could be utilized to control mosquito populations (Laven, 1967; Knipling et al., 1968). This concept, known as Incompatible Insect Technique (IIT), involves the release of large numbers of male mosquitoes infected with an incompatible strain of *Wolbachia* into the environment. These released males would compete for mating events, resulting in population depression. Theoretically, over the course of multiple releases, this could even result in the population being pushed completely out of a region (Dobson et al., 2002).

The most basic example of an applied IIT program is the translocation of an existing population into a region an incompatible endemic population. Translocation offers the benefit of needing little laboratory manipulation (Lin et al., 2013). However, it requires that two naturally occurring incompatible strains of *Wolbachia* are found within two mosquito strains capable of interbreeding.

The translocation method can be modified through the introduction of an introgressed line, where *Wolbachia* has been introduced into a new host through hybridization and outcrossing. The earliest examples of IIT utilized this technique. *Wolbachia* from a *Culex pipiens*

line was introgressed into a *Culex pipiens quinquefasciatus* line and used to suppress a *Cx. p. quinquefasciatus* population in Myanmar (Laven, 1967). Since then, multiple introgressed lines have been developed with the intent of controlling mosquito populations (Brelsfoard et al., 2008; Atyame et al., 2011). However, this technique limits potential *Wolbachia* strains to those that occur within a group of closely related mosquito species.

Lines artificially infected through cytoplasmic microinjection, although comparatively more labor intense, lack this limitation; as such, they have received a great deal of attention. Extensive work has gone into generating artificially infected *Aedes* mosquito lines (see Xi et al., 2005 as an example). And recently, an artificial *Anopheles* infection was generated (Bian et al., 2013). However, no such artificial lines have been produced in the *Culex* genus. *Culex* mosquitoes are vectors of pathogens such as West Nile Virus and Equine Encephalitis. I attempted to generate several artificial lines, including a novel *Culex* line, with the hopes that such lines could be utilized in the development of future IIT programs.

Materials and Methods:

Mosquito Lines

Six mosquito lines were used in this experiment. First, a wild-type *Aedes albopictus* "WC3" line infected with both the *w*AlbA and *w*AlbB *Wolbachia* types collected from Lexington, KY in the summer of 2014. Second, an *Aedes albopictus* "HT1" line originating from Houston, TX cleared of *Wolbachia* infection through repeated treatment with tetracycline and maintained in culture since 2001 (Dobson et al., 2001). Finally, we used a *Culex pipiens molestus* "CMM" line, an aposymbiotic CMT line, a wild type *Culex pipiens* "CPP" line, and an aposymbiotic CPT line

(See chapter 2 for information involving the origins and removal of infections from these four lines).

Collection and Handling of Mosquito Eggs for Microinjection

Oviposition behavior varies between *Culex* and *Aedes* mosquitoes. *Culex* eggs are oviposited directly on the surface of water, while *Aedes* eggs are typically laid on substrate adjacent to a water source. I therefore, modified the egg collection method depending on the genus.

For the WC3 and HT1 *Aedes* lines, a small plastic cup (Conex 163 mL clear portion container) lined with a moist piece of Anchor Paper brand germination paper was placed in a Bioquip 1450 BS collapsible cage containing mosquitoes. The cage was then covered with black fabric and mosquitoes were allowed to oviposit for 30 minutes. At which point the cup was removed and any oviposited eggs were used for manipulations.

For the CMM, CMT, CPP, and CPT *Culex* lines approximately ten adults of each sex were removed from the cage and placed in a lidded cup with 20 mL of bovine liver powder (NOW Foods) in solution (0.6g/L). This container was then covered with black fabric and left undisturbed for 1 hour, allowing the mosquitoes to oviposit. Adults were then aspirated out of the container and frozen. Resulting eggs were the used for manipulations.

Preliminary Method Development Trial

Preliminary and long-term observational data were collected to establish a baseline method for manipulating and desiccating *Aedes* mosquito eggs based on established methods.

Manipulated mosquito eggs were used as a control during injection experiments, and hatch rates were monitored as a measure of method success. Initial preliminary testing was conducted to examine the effects of egg desiccation in order to evaluat existing desiccation methods.

Additionally, preliminary trials were conducted to develop the best methodology for injecting *Culex* mosquito eggs. CMT eggs were manipulated, and their resulting hatch compared to unmanipulated CMT eggs and manipulated HT1 eggs. Later, CMT eggs were desiccated for multiple time periods and their resulting hatch examined in order to determine an optimal desiccation period. Finally, CMT eggs were injected with SPG buffer solution (Bovarnick et al., 1950), in order to examine injection induced mortality.

Handling and lining of Collected Mosquito Eggs for Microinjection

Collected eggs were allowed to melanize in the oviposition cup until they reached a light gray complexion, at which point they were transferred to moist filter paper using forceps. Eggs were then lined along the edge of the filter paper in units of 20, picked up using Scotch permanent double sided tape, and the tape placed on glass slides. Donor eggs were immediately covered with hydrated halocarbon oil and set aside. Recipient eggs were allowed to desiccate until approximately 10% had formed a dimple, approximately 3 minutes.

Microinjection and Maintenance of Mosquito Eggs

All three of the infected mosquito lines (WC3, CPP, and CMM) were used as donors for microinjection. Similarly, the three uninfected lines (HT1, CPT, and CMT) were used as recipients. However, not all potential pairings were conducted during the course of injections.

The *Ae. albopictus* line WC3 was injected into HT1 and CMT recipients. Cytoplasm from infected *Cx. p. pipiens* line CPP was injected into CPT and HT1 eggs. Finally, infected *Cx p. molestus* cytoplasm was injected into CMT eggs.

Donor cytoplasm was drawn using a Sutter Instruments 1.0mm width quartz glass needle in conjunction with a Narishige IM 300 Microinjector. Cytoplasm was then injected into recipient eggs until the eggs appeared fully hydrated (Figure 3.1). Once all 20 recipient eggs on the slide were injected, the slide was set aside and the eggs allowed to rest in oil for approximately one hour. Eggs were then transferred to moist filter paper, and the oil cover removed. Cleaned eggs were then washed into labelled Petri dishes and provided two drops of liver powder solution (60g/L).

Injected eggs were observed at 48 and 96 hours post-injection. Hatched individuals were transferred to small cups and allowed to develop. Any resulting pupae were transferred to test tubes to eclose. Resulting female adults were then transferred to small buckets and provided with newly eclosed uninfected males of the same species. Approximately seven days later, females were provided a mouse as a bloodmeal (IACUC protocol # 00905A2005) and an oviposition site.

After three days, eggs were removed and placed in a Pactiv hinged lid container with 400 mL of distilled water and 2.5 mL of liver powder solution (60g/L). Larvae were fed as needed until pupation, at which point they were treated identically to parental pupae.

After the first ovigenesis cycle, adults were placed in a -20˚C freezer for approximately 1 minute to reduce activity. They were then placed in centrifuge tubes and preserved in 200 proof ethanol until PCR analysis.

Verification of Infection through PCR

A general CO1 primer set was used as a control for each sample and presence of *Wolbachia* was verified through the use of the general *Wolbachia* primer Wol438 set (5'-CATACC TATTCGAAGGGATAG-3' forward, 5'-AGCTTCGAGTGAA ACCAATTC-3' reverse) (Folmer et al., 1994; Werren and Windsor, 2000). Additionally, a *Culex*-*Wolbachia* specific Orf7c primer set (5'-CCCACATGAGCCAATGACGTCTG-3' forward, 5'-TTGCTTGCTCAACACTTACACTT-3' reverse) was used to test for *w*Pip strains in injected *Aedes* mosquitoes (Sanogo and Dobson, 2004).

DNA was extracted using whole mosquitoes homogenized in 100 µL squash buffer (Gloor et al., 1991). Following extraction, 1 µL of each sample was amplified in 2 µL NEB 10X buffer, 0.5 µL dNTP (10mM), 0.5 µL primers, and 0.2 µL NEB Taq in a total volume of 20 µL. Amplifications occurred in a PTC-200 Thermal Cycler. Samples were denatured at 94˚C for 2 minutes, then cycled 38 times among 94, 55, and 72˚C for 30, 45, and 90 seconds respectively, followed by 72˚C for 10 minutes. A volume of 7 µL of each amplification product was separated on 1% agarose gel, stained with GelRed, and visualized using ultraviolet light.

Results:

Manipulation and Desiccation of *Aedes* Mosquito Eggs

Initial efforts focused on reproducing previously published *Wolbachia* injection method with *Ae. albopictus* as a means of assessing my injection technique. Initially the manipulation and alignment of newly laid eggs resulted in low hatch (Figure 3.2). Given these observations, the level of hydration in the filter paper on which the eggs were placed was increased.

Additionally, the tool used to manipulate eggs was changed from a brush to forceps. Subsequently, the average hatch increased to between 65% and 95% (Figure 3.2).

The desiccation of manipulated *Aedes* eggs reduced hatch. Eggs desiccated for approximately three minutes before being covered in hydrated halocarbon oil were observed to have an average hatch rate of 19%±7.2% SE compared to an observed 60%±18% hatch among manipulated but undesiccated eggs (Figure 3.3).

Microinjection of *Aedes albopictus* Eggs with Cytoplasm

Over the course of multiple experiments conducted over seven months, HT1 eggs were injected with WC3 cytoplasm. Of these 2405 eggs, 1.4% hatched. Of these 48 hatched eggs, 32 individuals survived to adulthood. Of the adults, 14 were female, and 12 successfully blood fed and produced eggs (Table 3.1).

In a separate series of experiments conducted over Two months, HT1 eggs were injected with CPP cytoplasm. Of the 817 HT1 eggs injected, 1.7% hatched. Of these 21 hatched larvae, 11 individuals reached adulthood. Of the adults, five were female, all of which successfully blood fed and produced eggs (Table 3.1).

Manipulation of *Culex* Mosquito Eggs

The average hatch for unmanipulated *Culex* eggs was examined over the course of three experiments and was observed to be 88.3±11.6%. Newly laid *Culex* eggs that were lined, adhered to double-sided tape, and covered with hydrated halocarbon oil had an observed

average hatch rate of 60.3±22.7%, which did not differ (p=0.129) from unmanipulated eggs (Figure 3.4).

The effect of desiccation on egg hatch rates was examined in a series of three experiments. In these experiments, manipulated eggs were desiccated for varying amounts of time. Desiccation was observed to be correlated to hatch rate, with increasing period of desiccation resulting in decreased hatch (Figure 3.5).

Once the effect of desiccation on hatch had been established, three experiments were conducted to determine the effect of injection induced trauma on hatch by desiccating eggs for three minutes and injecting them with SPG buffer. Desiccated but uninjected eggs were observed to have an average hatch rate of 59.4±12.9%. However, similar desiccated eggs injected with SPG had a lower observed hatch rate of 6.1±1.4% (p=0.002; Figure 3.6).

Microinjection of *Culex pipiens* Eggs with Cytoplasm

The 6.1% observed egg hatch rate was adequate to proceed to injections using cytoplasm. Two sets of experiments were conducted involving the injection of uninfected *Culex* eggs with intraspecific infected cytoplasm. In the first, 128 CPT eggs were injected with CPP cytoplasm; however, none of these eggs hatched (Table 3.2). In the next set of experiments, conducted over three months, CMT eggs were injected with CMM cytoplasm. Of the 492 CMT mosquito eggs injected with CMM cytoplasm, 8(1.6%) hatched. However, none of these reached adulthood (Table 3.2).

Additionally, two sets of *Culex* lines were injected with infected *Ae. albopictus* cytoplasm. In the first experiment, 90 CPT eggs were injected with WC3 cytoplasm; however,

none of these eggs hatched (Table 3.2). In the next set, conducted over four months, 471 CMT eggs were injected with WC3 cytoplasm. Of these injected eggs, 2(0.4%) hatched. Only one of these hatched eggs (50%) reached adulthood. However, the resulting individual was male (Table 3.2).

PCR Testing of Resulting Productive Female Lines

Injected eggs that survived to adulthood were tested to examine for the successful establishment of *Wolbachia* infection. PCR tests of the five HT1 individuals that survived injection with CPP cytoplasm revealed a ubiquitous infection among all five lines (Figure 3.7a). However, subsequent tests using a *Culex*-specific PCR assay demonstrated that none of the five were infected with the *w*Pip *Wolbachia* type from *Culex pipiens* (Figure 3.7b). Subsequently, the HT1 line used in these experiments was PCR tested for *Wolbachia* infection was found to be *Wolbachia*-infected. Specifically, the original HT1 line remained aposymbiotic, but the subline created for these experiments had become infected with *Wolbachia*. Due to the contamination of the HT1 line, the remaining survivors from the CPP injections were not tested. Additionally, survivors of WC3 injections were not tested, because it would be impossible to differentiate between the contaminating HT1 infection and any infection resulting from the artificial infections. No *Culex* individuals survived the injection process, and therefore no PCR assays were performed.

Discussion:

The development of artificially generated *Wolbachia* infections in mosquitoes offers a unique avenue for CI-mediated population control. While artificially infected lines have been developed in *Aedes* and *Anopheles*, there are no examples in *Culex*. While my experiments showed evidence of contamination and failed to generate an artificially infected line, my observations may help in the establishment of future *Wolbachia* lines in *Culex* mosquitoes.

Melanization of *Culex* eggs was observed to be markedly different from *Aedes* mosquitoes. While eggs collected from the HT1 and WC3 lines transitioned from white to black, the CPP, CPT, CMM, and CMT lines never melanized beyond a light gray color. This made gauging the age of the eggs difficult. In order to reduce the potential variation in the age of eggs, the period of time allowed for oviposition should be reduced. A one hour oviposition period was used in this experiment because 30 minutes failed to consistently produce eggs. With this in mind, a series of simple experiments could find the shortest period between 30 and 60 minutes that allows for consistent egg production.

It should also be noted that the elasticity of *Culex* eggs was different from *Aedes*. *Culex* eggs were more difficult to inject. Using the rate of dimpling as a marker for desiccation yielded eggs that were crushed by the needle rather than pierced by it. Even in cases where this did not occur, *Culex* eggs were more prone to bursting during the injection process than their *Aedes* counterparts. While I explored relying on desiccation time rather than physical appearance and saw a reduction in both crushed and burst eggs, the experiment was terminated before an optimal time could be found. I would suggest that future work begin exploring desiccation periods starting at approximately two minutes in high humidity environments.

The initial survival of my injected eggs ranged from an average of 0-1.68% across all injected groups. Additionally, between individual injection trials hatch varied between 0% and 11.5%. This peak is comparable to previous work in this lab, which showed a peak hatch rate of 12% (Xi et al., 2005). However, the average hatch rate is lower than other experiments, one of which produced an average hatch rate of 7.6% (Calvitti et al., 2010).

It is possible that low egg survival resulted from injections occurring too far along in development. However, this seems unlikely, as hatch was observed in eggs injected with SPG. More likely, the introduction of cytoplasm acted as an additional source of mortality.

Egg mortality resulting from injection trauma of eggs cannot be reduced easily. However, the post-injection procedure could be implemented to reduce mortality. *Culex* eggs are normally oviposited on the surface of the water, something which I attempted to simulate in the injected eggs. However, the presence of water may have acted as a means of fungi and bacteria to more easily invade the wound inflicted during injection and the damage to the chorion may have disrupted the water gradient within the egg. Taking this into account, I would suggest that early egg development, particularly the first 24 hours post-injection, take place on moist filter paper.

While this attempt to produce artificially infected mosquito lines failed due to contamination and low post-injection hatch, my results and observations can provide valuable insight into how others may succeed in the future. Most importantly among these is the need to increase post-injection hatch rates, which can likely be achieved through modulations in egg handling that optimize desiccation time, account for reduced egg elasticity, and reduce the risk of bacterial or fungal infection.

Tables & Figures:

	WC3 → HT1	CPP → HT1
Eggs Injected	2405	817
Eggs Hatched	48	21
Survived to Adulthood	32	11
Females	14	5
Produced Eggs	12	5

Table 3.1: Hatch and Development Results of Uninfected *Aedes* Eggs Injected with Infected Cytoplasm
Infected cytoplasm was injected into newly laid uninfected *Aedes* eggs. Resulting larvae were allowed to develop, and adult females were collected for breeding.
Injection set described as (Donor → Recipient)
HT1- Uninfected *Ae. albopictus*
WC3- Naturally infected *Ae. albopictus*
CPP- Naturally infected *Cx. p. pipiens*

	WC3 → CPT	WC3 → CMT	CPP → CPT	CMM → CMT
Eggs Injected	90	471	128	492
Eggs Hatched	0	2	0	8
Survived to Adulthood	0	1	0	0
Females	0	0	0	0
Produced Eggs	0	0	0	0

Table 3.2: Hatch and Development Results of Injected *Culex* Eggs

Infected cytoplasm was injected into newly laid uninfected *Culex* eggs. Resulting larvae were allowed to develop, and adult females were collected for breeding.

Injection set described as (Donor → Recipient)

WC3- Naturally infected *Ae. albopictus*

CPP- Naturally infected *Cx. p. pipiens*

CMM- Naturally infected *Cx. p. molestus*

CPT- Uninfected *Cx. p. pipiens*

CMT- Uninfected *Cx. p. molestus*

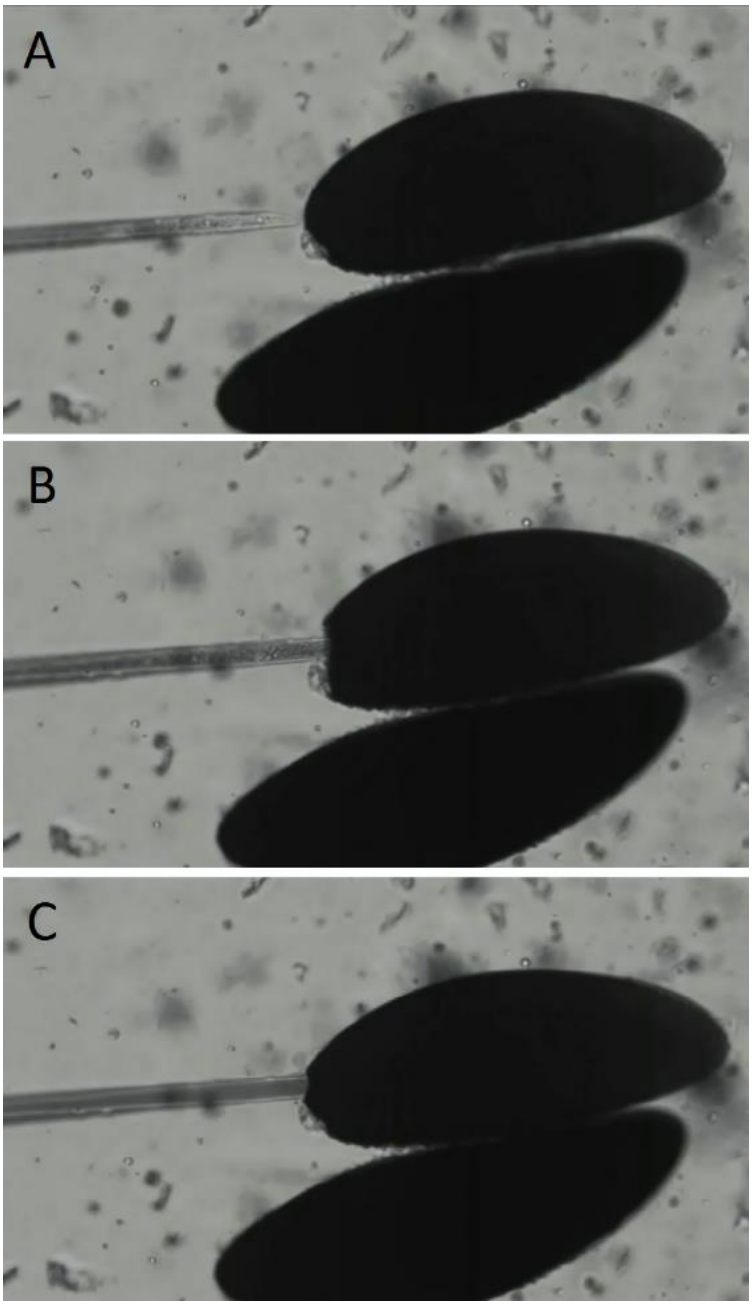

Figure 3.1: *Culex* Mosquito Egg Injection Process
Recipient eggs were innoculated with *Wolbachia*-infected cytoplasm using microinjection. The images shown are of an egg prior to (A), at the beginning of (B), and at the end of injection process (C).

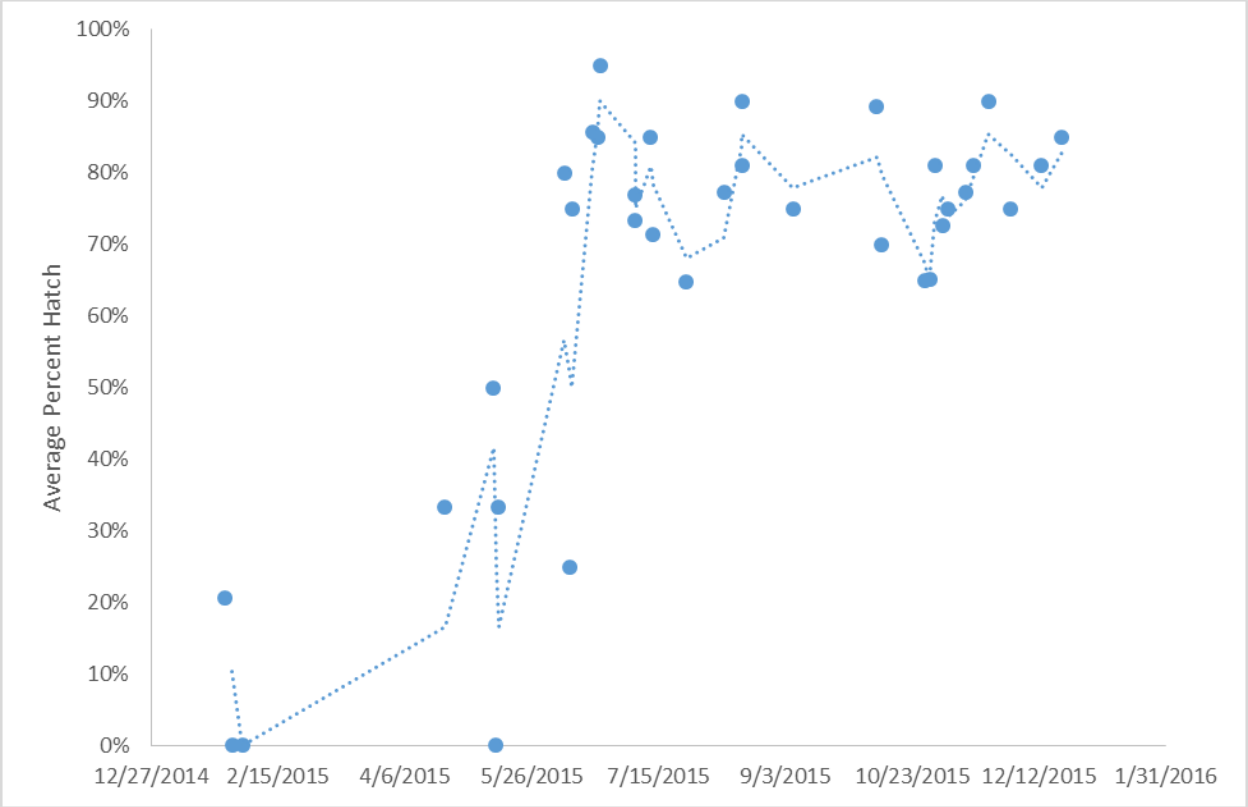

Figure 3.2: Percent Egg Hatch of Manipulated Eggs over Time

The hatch rate for manipulated *Aedes* eggs was initially low. However, it increased as more injection trials were conducted. Hatch rate for manipulated eggs based on 20 manipulated, but uninjected, eggs run as a separate control alongside each set of injected eggs. Dotted line represents running average hatch rate of manipulated eggs.

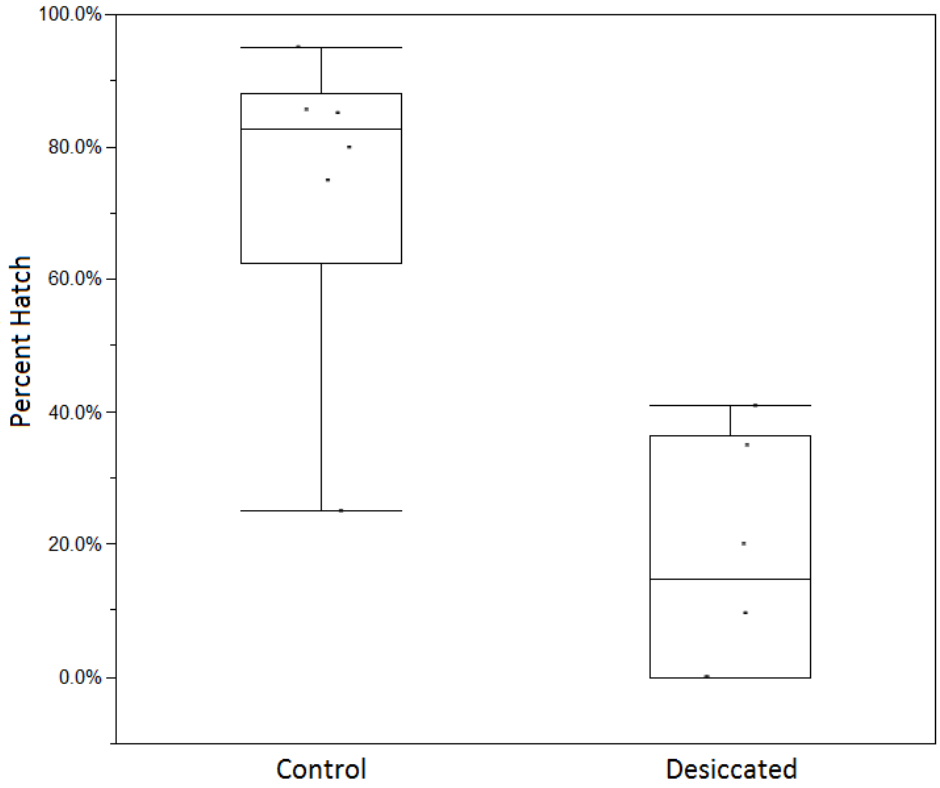

Figure 3.3: Box Plot of Hatch Rate for Desiccated and Undesiccated *Aedes* Eggs
Six replicates of 20 *Aedes* mosquito eggs were either desiccated or left untouched. Desiccation of mosquito eggs was observed to result in reduced hatch rate.

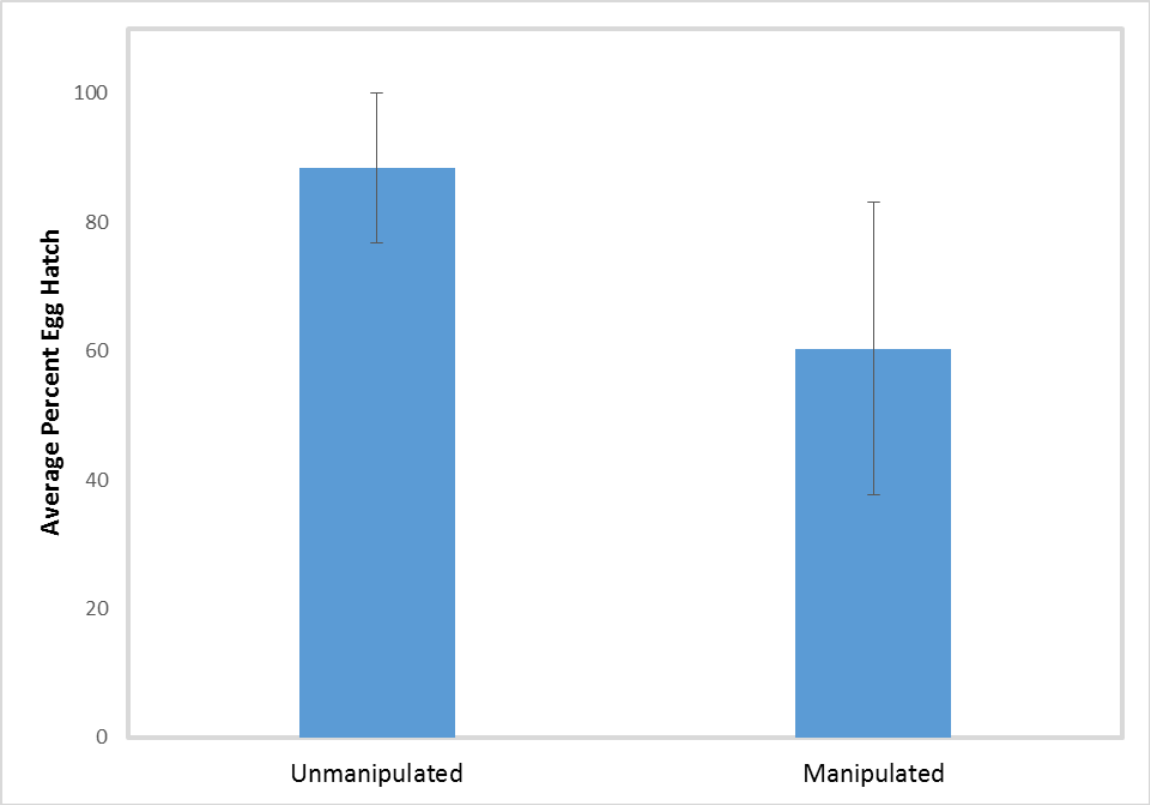

Figure 3.4: Average Egg Hatch of Manipulated *Culex* Eggs Relative to Unmanipulated Eggs
Three replicates of 20 recently laid *Culex* eggs were either manipulated or left untouched. Manipulation of *Culex* eggs had no observed effect on average hatch rate.
Results are displayed as Avg ± St Error.

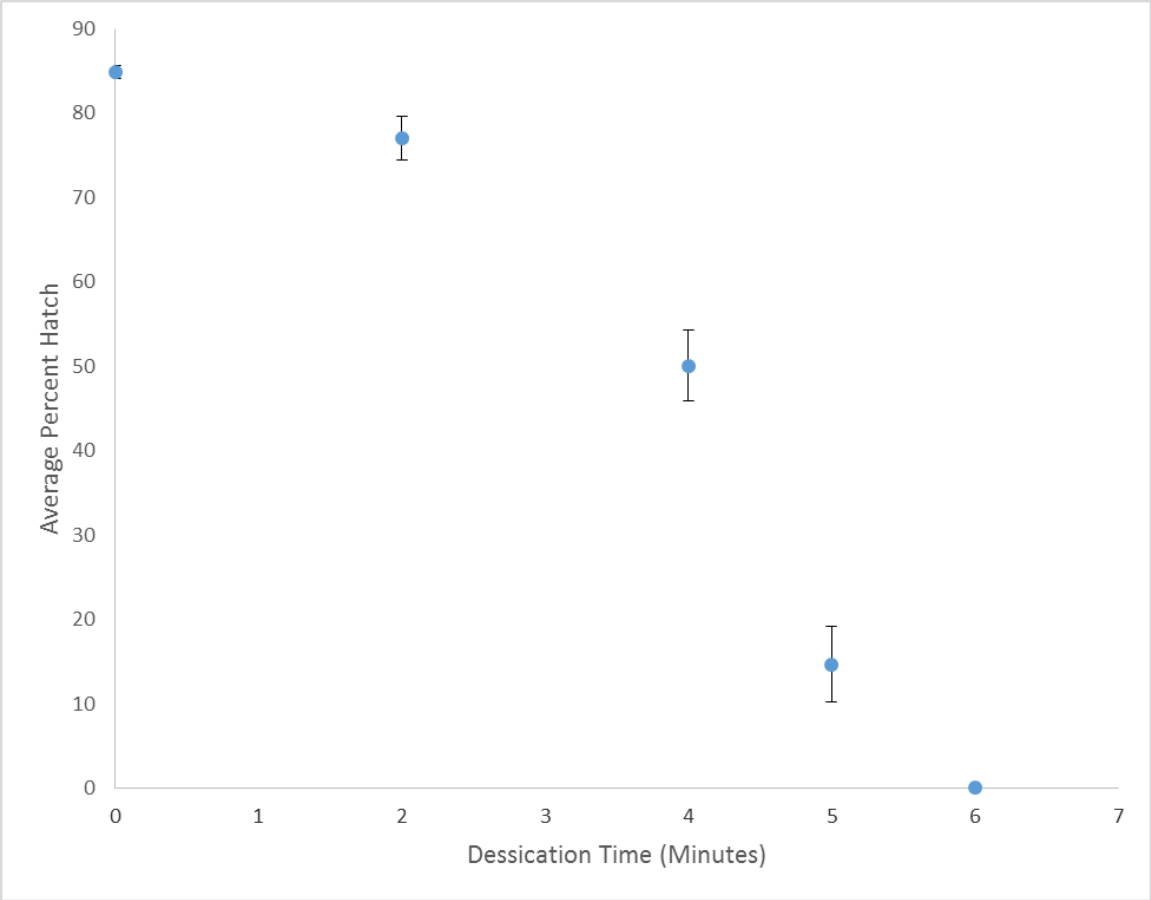

Figure 3.5: Average Percent Egg Hatch of Culex Eggs Desiccate for Variable Periods of Time
Three replicates of 20 Culex mosquito eggs were desiccated for periods of time ranging from zero to six minutes. Increased period of desiccation was observed to result in decreased average hatch rate. Results are depicted as Avg ± St Error.

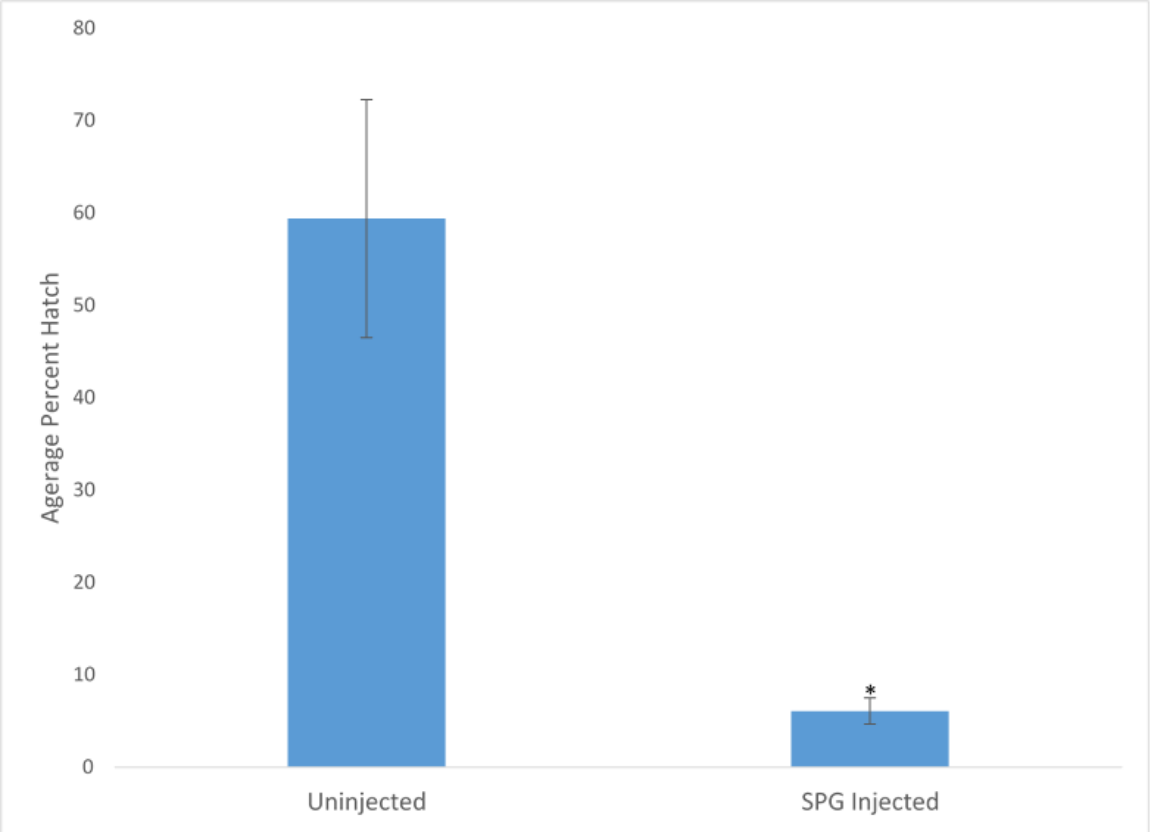

Figure 3.6: Average Percent Egg Hatch of Culex Mosquito Eggs Injected with SPG Buffer
Three replicates of 20 manipulated and desiccated *Culex* eggs were either injected with SPG buffer or left untouched. Injection of eggs with SPG solution was observed to reduce hatch relative to uninjected eggs ($p=0.002$).
Results are depicted as Avg ± St Error.
* Represent statistical difference ($p \leq 0.05$)

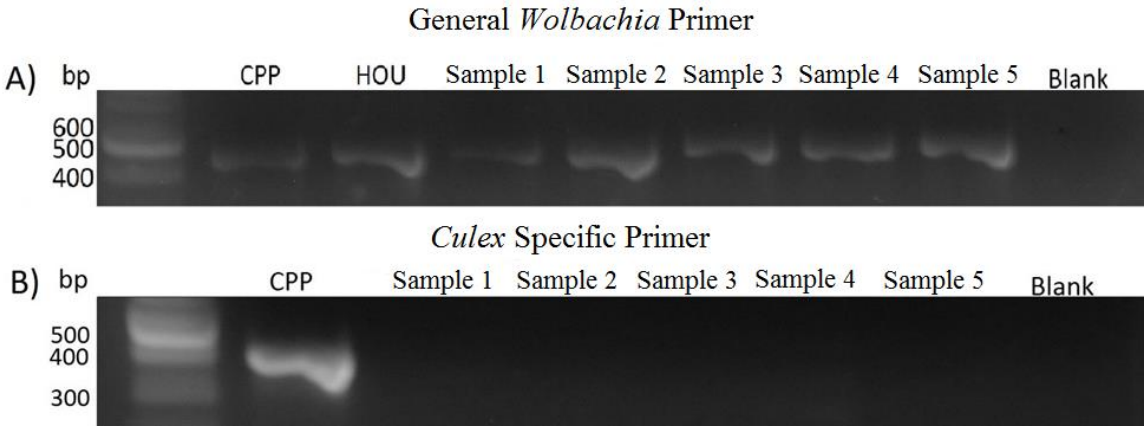

Figure 3.7: Ploymerase Chain Reaction Results of Adult Female HT1 Mosquitoes Injected with Infected Cytoplasm

Polymerase chain reactions were conducted on whole adult females resulting from the injection of uninfected *Ae. albopictus* (HT1) egg injected with naturally infected *Cx. p. pipiens* (CPP) cytoplasm using the general *Wolbachia* primer WOL438 (A) and the Culex-Wolbachia specific primer ORF7C (B). Resulting bands showed the presence of an infection in all samples (A). However, testing with the *Culex Wolbachia* specific primer Orf7C showed no presence of CPP *Wolbachia* (B).

Research Conclusions

The mechanisms by which *Wolbachia pipientis* manipulates its host are poorly understood, but because it is a reproductive parasite, it is useful as a potential means of population control. Taking this into account, I began my research with two goals in mind. The first was to better understand the relationship between two members of the *Culex pipiens* complex and their *Wolbachia* infections. The second was to use cytoplasmic injection to establish a novel line of transinfected *Culex* mosquitoes.

My examination of the relationship between *Culex pipiens pipiens*, *Culex pipiens molestus*, and their respective *Wolbachia* infections yielded intriguing results. Crossing experiments between the two sub-species showed complete unidirectional incompatibility, with *Cx. p .molestus* males acting as the source of incompatibility. Analysis of loss of *Wolbachia* infection status showed no effect on either hatch rate or egg production. Further analysis of how hybridization affected these metrics showed that only one cross, infected *Cx. p. pipiens* females crossed with uninfected *Cx. p. molestus* males resulted in a reduced hatch rate.

My work in establishing artificial *Wolbachia* infections in *Culex* failed to produce a successfully transinfected line. Only one of the *Culex* eggs injected with foreign cytoplasm hatched, and no line resulted from it. Despite this, I noted several potential means to increase egg hatch in future experiments.

The ultimate goal of these experiments was to better understand *Wolbachia*'s relationship with its host and develop new lines of artificially infected *Culex* mosquitoes for use in mosquito control. While I was unable to produce any transinfected lines, my observations on how *Culex* mosquitoes and *Wolbachia* interact may prove useful for future research by providing us with a better understanding of how *Wolbachia* manipulates its host.

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2012 to 2014 – Temporary Research Worker; Insect Production and Research Lab, DuPont Pioneer

2008 to 2012 – Laboratory Assistant; Pesticide Toxicology Laboratory, Iowa State University

Memberships:

Entomological Society of America, 2009 to Present

Teaching:

Teaching Assistant. Fall 2016. ENT 320: Horticultural Entomology.

Scientific Publications:

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McNamara, Timothy and Dobson, Stephen. 2016. The Removal of *Wolbachia* from Two Medically Important Mosquitoes and its Effect on Reproduction. Parasites & Vectors. (In Prep)

Stamper, C., Jackson, K., McNamara, T., Skidmore, A., McCord, J., Ferguson, B., Hilario, A., Cerenka, J., Layman, M., Bredeson, M. 2015. A tale of three cities: Student perspectives on a

hybrid live-distance IPM class. North Central Branch Entomological Society of America Conference. Manhattan, KS.

Presentations:

McNamara, Timothy. 2016. Zika in Kentucky: What You Need to Know. Kentucky Nursery & Landscape Association Summer Retreat. Frankfort, KY.

McNamara, Timothy. 2016. Zika Risk and Control in Kentucky. University of Kentucky Turf Research Field Day. Lexington, KY

McNamara, Timothy. 2015. Characterization of Cytoplasmic Incompatibility Relationships in Medically Important Mosquitoes. Ohio Valley Entomological Association. Lexington, KY.

Outreach/Extension:

McNamara, Timothy. 2016. Making Professional Connections during Secondary Education. National FFA Organization: Olathe, KS Chapter. Olathe, KS

McNamara, Timothy. 2016. The Role of Insects in Horticultural Systems. Olathe North High School. Olathe, KS

McNamara, Timothy 2016. Professional Opportunities and Development in Academia and Scientific Industry. Olathe North Distinguished Scholars Program. Olathe, KS

Baker, Adam, McNamara, Timothy, Mach, Bernadette. 2016. Monarch Conservation through Waystation Maintenance. Sustainable Berea: Celebrate the Harvest. Berea, KY.

Media:

Mach, Bernadette and McNamara, Timothy. December 2016. Bee Friendly Landscape Plants. Greenhouse Production News.

McNamara, Timothy, Mach, Bernadette. August 2016. Monarch Monitoring. Madison County Extension Newsletter. Richmond, KY

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