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# The Effects of Juvenile Exposure to Sub-lethal Concentrations of Pesticides on the Adult Reproductive Biology of the Male Southern House Mosquito, *Culex quinquefasciatus*

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THE EFFECTS OF JUVENILE EXPOSURE TO SUB-LETHAL  
CONCENTRATIONS OF PESTICIDES ON THE ADULT REPRODUCTIVE  
BIOLOGY OF THE MALE SOUTHERN HOUSE MOSQUITO, *CULEX*  
*QUINQUEFASCIATUS*

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
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Master of Science

in

The Department of Entomology

by  
Shiloh Dean Judd  
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## Abstract

Mosquitoes transmit pathogens capable of causing significant diseases, the worst of which can permanently disfigure or kill. Mosquito control efforts can be strengthened by better understanding all aspects of mosquito biology, but there is deficient information regarding sperm production in male mosquitoes. Understanding the sperm production and capacity of vector species may reveal new control strategies.

The goal of this research was to determine if larval exposure to sub-lethal concentrations, defined as killing less than half of the sample population, of pesticides negatively impacts the adult male reproductive health of the southern house mosquito, *Culex quinquefasciatus* Say. This study quantified sperm counts for wild and lab reared (Sebring) *Cx. quinquefasciatus*. The Sebring colony produced more sperm than the wild collected mosquitoes. After baseline sperm counts were determined, each population was exposed to sub-lethal quantities of the larvicides spinosad, *Lysinibacillus sphaericus*, and methoprene, and the herbicides glyphosate and atrazine. Larvae were exposed during their third instar and remained in an environment with pesticide until eclosion. Adult males were dissected at one, four, seven, and fourteen days post eclosion.

The reproductive tracts were removed and the sperm diluted, and the sperm were counted in subsamples. Sperm counts for the Sebring colony were significantly reduced after the spinosad and *L. sphaericus* treatments, and significantly increased after the methoprene treatment. Sperm counts for the wild collected mosquitoes experienced no decrease from any treatment, but they did experience significant increase after the methoprene treatment. Wing lengths (as a proxy for body size) were measured for all experiments, but the treatments had no observed effects on body size. These results show that exposure to some chemicals during larval development can alter spermatogenesis in *Culex quinquefasciatus* males.

# **Chapter 1: Literature Review**

## **1.1 Mosquitoes and Pathogens**

Mosquitoes are hematophagous insects of public health concern due to their competence as vectors of disease-causing pathogens. When an infectious mosquito bites a susceptible host, the infectious agent may travel from the mosquito into the new host. Hundreds of infectious pathogens have been found in mosquitoes, but just under 100 are known to cause disease in humans (Karabatsos 1985). Vector borne disease incidence in the United States has been increasing over the past decade, and while much of that increase is due to tick borne diseases, nine new mosquito borne diseases were detected for the first time between 2004 and 2016 (Rosenberg et al. 2018). The deadliest mosquito borne disease today is malaria and it is responsible for the deaths of hundreds of thousands of people every year (WHO 2016). An estimated 3.4 billion people living in malaria endemic countries are considered at risk of contracting the disease (Hay et al. 2004). While global malaria risk has decreased as intervention strategies have been implemented, current climate change modeling shows the potential for a substantial expansion throughout the world (Caminade et al. 2014).

The most prolific mosquito borne disease affecting human health today is dengue fever. One group predicts that 390 million cases of dengue infection occur each year with nearly one million of those cases becoming symptomatic (Bhatt et al. 2013). Both malaria and dengue are endemic throughout tropical areas, but there are other potentially life-threatening mosquito borne diseases in other regions, including those in temperate climates. West Nile virus has caused over 2,000 deaths in the United States since its introduction in 1999 (CDC 2016). Despite having an effective vaccine for the Japanese encephalitis virus, an estimated 68,000 cases of Japanese encephalitis occur annually in Asia (Campbell et al. 2011). Important vectors from the genera



*Aedes*, *Anopheles*, and *Culex*, responsible for the spread of dengue, malaria, and West Nile respectively, are distributed globally with many species expanding in range (Tatem et al. 2006). The presence of mosquitoes in shared human habitats and their capacity for pathogen transmission justifies their importance at the forefront of public health entomological research.

## **1.2 *Culex quinquefasciatus***

The southern house mosquito, *Culex quinquefasciatus* Say, is an urban and sylvatic species found in tropical to sub-tropical areas throughout the world and is a competent vector of disease causing pathogens. Females of this species seek out organically rich, ephemeral, or permanent pools of water for oviposition. This habit helps guarantee close proximity to humans as we provide them with these exact habitats via septic ditches, sewers, and other manmade containers that collect water (Chaves et al. 2009). Female *Cx. quinquefasciatus* typically seek out birds for blood meals, the reservoir hosts of the West Nile and St. Louis viruses, but commonly blood feed on other vertebrates (Molaei et al. 2007). This species is widespread throughout the southern United States in both rural and urban environments (Vinogradova 2000). Within its range in the United States, it is a competent vector of St. Louis virus, West Nile virus, and the canine heartworm, *Dirofilaria immitis* (Lai et al. 2000, Godsey Jr et al. 2005, Reisen et al. 2005). In other parts of the world it transmits *Plasmodium relictum*, the causative agent for avian malaria, and *Wuchereria bancrofti*, the nematode responsible for lymphatic filariasis (Bartholomay et al. 2010). Localized suppression of this mosquito can effectively suppress disease incidence as was found in India with lymphatic filariasis (Ramaiah et al. 1992), and in California with West Nile virus (Carney et al. 2008). The control costs of this mosquito combined with the medical costs caring for those afflicted with West Nile fever were almost \$3 million dollars for just one outbreak in Sacramento County, CA (Barber et al. 2010). The annual costs associated with lymphatic filariasis amount to hundreds of billions

of dollars throughout the world (Keating et al. 2014). The combined threat of its global distribution and competence as a vector of disease-causing pathogens has warranted *Cx. quinquefasciatus* a species of interest in both control and research.

### **1.3 Reproductive Biology of Male Mosquitoes**

The reproductive development of male mosquitoes has been well documented, but the specific roles of hormones in this development have not. The primordial tissues which differentiate and become testes begin lengthening during the first instar, then lengthen dramatically during the fourth instar and pupal stage (Horsfall and Ronquillo 1970). The appearance of gonads begins in the late larval stages with spermatocytes visible in the fourth instar and pupal stage (Dumser 1980). While mature sperm are not stored in the seminal vesicles during the pupal stage, they are present in the testes of pharate adults (Jones 1967). The adult male reproductive system is comprised of paired testes, seminal vesicles, and accessory glands. These are connected one to another by the vasa efferentia and the vasa deferens. The system terminates posteriorly with the aedeagus. External claspers are used to grasp the female to aid in stabilizing copulation which most frequently occurs in flight (Jones 1968). Both ecdysteroids and juvenile hormone have been shown to inhibit and advance testicular growth in various insects (Dumser 1980, Raikhel et al. 2005), but without explicit experiments on mosquitoes it cannot be said how these substances may or may not effect male mosquito reproductive development.

Mosquito sperm production increases with age and can be manipulated by environmental conditions or chemical treatments. The first study to quantify sperm in *Aedes aegypti* found that body size significantly impacted sperm count with large males having more sperm than small males, an observation that held true from the ages of one day (mean sperm  $\pm$  SEM, small:  $2,843 \pm 159.3$ , large:  $3,714 \pm 197.8$ ,  $P = 0.002$ ) to ten days (small:  $7,306 \pm 563.8$ , large:  $9,704 \pm 416.8$ ,  $P$

= 0.004) post eclosion (Ponlawat and Harrington 2007). A follow up study found that larger and older *Ae. aegypti* males transferred more sperm to females than their smaller and younger counterparts (Ponlawat and Harrington 2009). *Aedes aegypti* were sampled from four different environments in Australia to measure the effects of climate on fecundity parameters. It was found that neither sperm production nor insemination rate differed significantly with origin alone, but males reared at high temperatures (producing smaller mosquitoes) had significantly higher sperm counts than those at low temperatures (producing larger mosquitoes) (35 °C, 18 °C, P = 0.02) (Bader and Williams 2012). The apparent contradiction between these two sets of experiments, one stating that larger males had higher sperm counts and the other stating that high temperature reared small bodied males had higher sperm counts, illustrates that both temperature and density can affect sperm count in *Ae. aegypti*.

A similar experiment to the first study quantifying *Ae. aegypti* sperm found that *Aedes albopictus* males produce sperm in a similar fashion. Large *Ae. albopictus* males produce more sperm than small males and sperm count increases over time (Hatala et al. 2018). However, unlike small *Ae. aegypti*, small *Ae. albopictus* males stopped producing sperm after 10 days post eclosion (dpe). The first study to quantify *Anopheles* sperm also looked at the effects of radiation on sperm count. *Anopheles arabiensis* irradiated as pupae with 70 and 120 Gy had significantly lower sperm counts than controls at six dpe; no effect on sperm count was observed for adults receiving the same irradiating treatment (Helinski and Knols 2009). *Anopheles arabiensis* sperm was also quantified after being irradiated with 70 Gy, treated with the organochloride dieldrin, or both. Irradiated males had significantly lower sperm counts at two dpe and did not produce more sperm by day six. Dieldrin treated males had significantly lower sperm counts at day two, but recovered to control level sperm counts by day six. The combined treatment also had lower day two sperm

counts, but was again normal by day six (Damiens et al. 2013). These five studies are the only published accounts of sperm quantification and account for only three mosquito species from two genera. The results of these papers are generally in agreement, that mosquito sperm increases with age and that density, temperature, and chemical exposure can alter normal sperm production.

Many mosquito species swarm in large groups for mate selection and these swarms are typically male dominated, with females entering alone and leaving mid copulation (South and Catteruccia 2016). Research shows that both pheromone signaling (Cabrera and Jaffe 2007) and wing beat frequency (Ritchie and Immonen 2010) contribute to the formation of and mate selection within these swarms. The detection of a conspecific female wing beat frequency causes significant reductions in flight speed for male *Cx. quinquefasciatus*, suggesting a controlled and precise mate selection process (Gibson 1985). While it was explained previously that larger males are capable of transmitting more sperm to females, larger males are not always the most sexually competitive. An experiment with *Anopheles gambiae* demonstrated that males of mid-range size, lipid, glycogen, and sugar reserves were significantly more successful at mating than either large or small males with corresponding energy reserves (Ng'habi et al. 2008). It is not well understood how males select swarm locations, but *Anopheles* males appear to prefer swarming above substrates with light-dark contrast (Diabate and Tripet 2015) and some *Aedes* males swarm near the females' preferred blood hosts (Hartberg 1971). *Culex quinquefasciatus* swarms are composed of both male and female mosquitoes and, while their in-swarm behaviors have been well documented, swarm site selection stimuli (if any) is still unknown (Gibson 1985).

Mosquito mating fidelity is variable among species, but it is common to see reports of polygynous males and monandrous females. Male mosquitoes are polygynous, or are at least physiologically capable of being so (the rate of multiple successful mating events in the wild is

unknown), and are capable of producing sperm throughout their field-relevant lifetime (Yuval 2006, Ponlawat and Harrington 2007). Monandry is practical for a female mosquito as one mating event provides her with enough sperm for her lifetime and allows energy to be spent on host seeking and oviposition rather than mating (Craig 1967). Monandry in females is also regulated by male accessory gland (MAG) substances with matrone as one of the leading MAG substances responsible for mating refractoriness (Sirot et al. 2008). Monandry in many *Anopheles* mosquitoes is also regulated by mating plugs which provide a physical barrier to subsequent inseminations (Giglioli and Mason 1966). While healthy virgin males are well stocked with MAG substances, their supply decreases with each consecutive mating event and while MAG substances can be replenished over time (Foster and Lea 1975), a MAG substance-depleted male may be unable to induce mating refractoriness in a female (Dickinson and Klowden 1997). It is important to note that the refractory effect of MAG substances are not immediate in some species. Most *Ae. aegypti* females become refractory within a few hours of mating, but 3% of females may still be polyandrous after 20 hrs post-mating. (Degner and Harrington 2016). This laboratory-based study is corroborated by wild-collected female *Ae. albopictus* which oviposited eggs with mixed paternity (Boyer et al. 2012). Early studies of *Culex* mosquitoes showed that females do experience mating-induced monandry (Kitzmilller and Laven 1958) and that several hours are required for the effect to become complete (Bullini et al. 1976). As far as it has been observed, male mosquitoes are polygynous and female mosquitoes are primarily monandrous with time-dependent exceptions.

#### **1.4 Juvenile Hormone**

Juvenile hormone (JH) is an insect sesquiterpenoid synthesized primarily in the corpora allata (CA) with a complex receptor and mode of action process (Goodman and Cusson 2012). JH is a key regulator of the molting process, and a more detailed review of this process will be covered

later. Feeding stimulates JH synthesis in the CA, a process that follows the mevalonate pathway, but instead of producing cholesterol as it does in vertebrates, it diverts and follows what is known as the JH branch (Bellés et al. 2005). The mevalonate pathway, in insects, begins with Acetyl-CoA and undergoes 13 enzymatic transformations before the final product, JH (Nouzova et al. 2011). JH is not stored in the CA, but is released directly into the hemolymph and, as a lipophilic substance, it requires carrier proteins for target site dispersal (Trowell 1992). The search for JH target sites has spanned several decades and has only recently produced compelling evidence. The *Methoprene-tolerant* (*Met*) gene proteins bind JH and JH analogs, are only present in certain tissues, and are only present within the nuclei of the cells of these tissues (Pursley et al. 2000). *Met* has been confirmed as a key receptor of JH in adult female *Ae. aegypti*, affecting gene expression for reproductive regulation (Zou et al. 2013). However, JH and JH analogs do not bind exclusively with *Met*. The nuclear receptor Ultraspiracle also binds JH in a dose dependent manner, leading to conformational changes in the receptor, which then leads to a transcriptional response (Jones et al. 2001).

Despite the confirmation that these two receptors bind JH and produce transcriptional changes, there is evidence that other receptors may play a part and their roles have not been explored conclusively. It is likely that these and other receptors work together as a JH receptor complex (Jindra et al. 2013). Beyond nuclear receptors, it is proposed that JH acts at the cell membrane level (Wheeler and Nijhout 2003). In *Drosophila*, JH acts on the cell membrane level of the male accessory glands via the Protein Kinase-C signaling pathway, initiating the synthesis of post-copulatory proteins (Yamamoto et al. 1988). JH is received through this same membrane pathway in the ovarian follicle cells of *Rhodnius prolixus* (Hemiptera: Reduviidae), resulting in constriction of these cells allowing for yolk movement and oocyte development (Sevala and Davey

1993). In *Ae. aegypti* the pathway initiated by membrane receptors of JH has been shown to modify *Met* activity, suggesting that membrane and nuclear receptors work in concert to bring about transcriptional changes (Liu et al. 2015). The search for JH receptors continues and many questions about the mode of action of JH, whether at the nuclear or membrane receptor level, remain to be answered (Jindra et al. 2015).

Sexual maturation of both female and male mosquitoes relies in part on JH. While ecdysteroids regulate most of the reproductive processes in female mosquitoes, JH plays an essential role in priming the fat body so that ecdysteroids can initiate yolk protein synthesis (Raikhel et al. 2005). JH also regulates ovarian nutrient allocation during previtellogenesis (Noriega 2004) and facilitates follicular resorption during times of nutritional stress (Clifton and Noriega 2011). Mosquito ovaries are also capable of producing their own JH from farnesoic acid (Borovsky et al. 1994a). Less is known about the role of JH in male reproductive development, but key discoveries have been made. MAGs produce many proteins and other substances involved with reproduction and are capable of producing and secreting JH (Borovsky et al. 1994b). The transfer of MAG substances, including JH, have been shown to influence pre-oviposition behaviors of female mosquitoes such as oviposition site selection (Yeh and Klowden 1990), host seeking behavior (Fernandez and Klowden 1995), and mating refractoriness (Klowden 2006). Newly-mated female mosquitoes have increased JH titers, more ovarian lipids, a reduction in follicular resorption, and increased fecundity (Clifton et al. 2014). Adult male mosquitoes treated with a JH mimic were able to mate with more females than untreated males, and males with depleted MAGs were able to overcome mating refractoriness more quickly than controls (Ramalingam and Craig 1977). While there are many experiments showing the effects of JH and MAG substance transfer during mating, it has been difficult to isolate the JH affects from the other

MAG substances. At this time there is no exclusive evidence that the maturation of sperm or other reproductive traits in male mosquitoes are controlled by JH (Nouzova et al. 2018). Female mosquitoes are prime targets for mosquito control efforts because they transmit deadly pathogens and lay eggs. Understanding their reproductive biology has been a research priority. However, as our understanding of vector endocrinology increases, the need for more research on the roles of JH, and its synthetic analogs, in both male and female mosquitoes increases (Ferguson et al. 2005).

### **1.5 Effects of Larval Habitat Conditions on Adult Development**

The rate of development from egg to adult as well as the size and fecundity of the adult mosquito is dependent upon the environmental conditions of the larva's aquatic habitat. After a mosquito egg hatches, the larva molts through four instar stages, pupates, and emerges as an adult. Temperature has a direct correlation with adult body size and rate of development. As the temperature of the larval habitat increases, the rate of development also increases, but body size decreases (Mohammed and Chadee 2011). Inversely, as the temperature of the larval habitat decreases, the rate of development decreases, but body size increases (Rueda et al. 1990). Adult body size is also dependent upon larval density. High density larval conditions induce food scarcity and produce smaller bodied adults, and low density larval conditions enable food abundance and produce larger bodied adults (Lyimo et al. 1992). Insufficient larval diet and high density conditions can also reduce fecundity (Telang and Wells 2004). All of these variables (temperature, density, and diet) interact simultaneously in the field and the interplay of these effects are only partially understood through laboratory experiments (Couret et al. 2014). Larval conditions must be considered and controlled for when evaluating the development rate, body size, or fecundity of adult mosquitoes.



## 1.6 Herbicide Contamination of Larval Habitats

The aquatic habitats of larval mosquitoes can be unintentionally contaminated by chemicals, such as herbicides. The two most commonly used conventional herbicides in the United States, applied to residential, commercial, and agricultural properties, are glyphosate and atrazine with 180 and 73 million pounds of active ingredient, respectively, applied in 2007 (Grube et al. 2011). Glyphosate is a post-emergent herbicide which inhibits amino acid synthesis in the shikimate pathway, causing an excess of shikimic acid which leads to plant death by the diverting of important resources away from necessary biological functions (Amrhein et al. 1980). Atrazine is a pre and post emergent herbicide which starves plants by inhibiting electron transport during photosynthesis (Dayan et al. 2000). As both of these chemicals have been found in larval mosquito habitats, studies have been undertaken to evaluate their effects on mosquitoes. *Aedes aegypti* larvae treated with atrazine at 5 ppm had significantly higher emergence rates and a reduction in male ratio bias when compared to glyphosate treated (5 ppm) and control larvae (Bara et al. 2014). *Aedes aegypti* larvae pre-exposed to atrazine at either 1, 10, 100, or 1000 µg/l for 48 hrs had reduced sensitivity to the pesticide *Bacillus thuringiensis israelensis*, demonstrated by a 75% decrease in mortality (Boyer et al. 2006). A similar experiment showed that *Ae. aegypti* larvae exposed to atrazine at 1 or 10 µg/l for 48 hrs became significantly less susceptible to the larvicide temephos, apparently due to an observed increase in P450 monooxygenase activity (Jacquet et al. 2015).

Atrazine has been shown to increase densities of microbial organisms in freshwater ecosystems (Muturi et al. 2013). This provides a more abundant food source for larval mosquitoes and creates an environment suitable for producing larger and healthier adult mosquitoes (Boyer et al. 2006). However, none of these laboratory based studies take into account the importance of soil

and water composition or other contaminants as synergists in arthropod exposure to atrazine. Water quality, soil type, and co-contaminants can positively or negatively mitigate the effects of atrazine on arthropods (Liang and Lichtenstein 1974). While none of the above studies found any significant effects on mosquitoes after glyphosate treatments, other arthropod-glyphosate interactions have been observed. Significant negative sub-lethal effects such as reduced prey consumption, fecundity, and web building were observed in the spider *Alpaida veniliae* (Araneae: Araneidae) after glyphosate treatments (Benamú et al. 2010). The soybean pest predator *Chrysoperla externa* (Neuroptera: Chrysopidae) suffered similar population reducing sub-lethal effects, e.g., reduced fecundity and fertility in females (Schneider et al. 2009). Glyphosate and atrazine are abundantly used herbicides which can contaminate larval mosquito habitats, and while some interactions between these chemicals and mosquitoes are understood, many are not.

## **1.7 Mosquito Larvicides**

While some chemicals are incidentally introduced into larval mosquito habitats, others are added intentionally, such as larval control pesticides (larvicides). Larvicidal compounds may be traditional contact toxins (Ali et al. 1999), ingested bacterial byproducts (Federici et al. 2003), insect growth regulators (Khan et al. 2016), suffocating water surface oils (Harbison et al. 2015), or a combination of some of the above. This review will focus on three larvicides: spinosad (contact or ingested toxin), *Lysinibacillus sphaericus* (ingested bacterial byproduct), and methoprene (insect growth regulator).

### **1.7.1 Spinosad**

Spinosad is a pesticide with mosquitocidal properties derived from fermented metabolites of the bacteria *Saccharopolyspora spinosa*. The two active metabolites utilized in pesticide formulations are spinosyn A and spinosyn D, which can be ingested or absorbed through the cuticle

to reach their target sites (Jiang and Mulla 2009). The primary target site of spinosad is the nicotinic acetylcholine receptor where it binds in a novel location, appearing to have no interaction with target sites of other pesticides. Post-binding symptomology observed in the central nervous system caused by neuron hyper-excitation includes convulsions, paralysis, and death (Salgado 1998). It also appears to act secondarily as a GABA neurotransmitter agonist (Orr et al. 2009). Spinosad is generally considered a new pesticide, especially in mosquito control. It only became recognized as a valid product for use against mosquito larvae in 2007 (Hertlein et al. 2010). While the efficacy of spinosad varies by species, it has been found effective against the larvae of the *Aedes*, *Anopheles*, and *Culex* genera (Bond et al. 2004, Jiang and Mulla 2009).

The sub-lethal effects of spinosad on mosquitoes have not been thoroughly researched, but studies using other model insects can potentially provide some insight. One study that did measure the sub-lethal effects of spinosad on mosquitoes found that surviving *Ae. aegypti* exposed to LC<sub>50</sub> concentrations had sex-biased results. Surviving females had significantly larger bodies than control females while males had significantly smaller bodies than control males. Results from the same study showed treated females laid more eggs than controls, but had lower egg hatch percentages (Antonio et al. 2009). Larvae of the eastern bumble bee, *Bombus impatiens* (Hymenoptera: Apidae), exposed to varying sub-lethal concentrations of spinosad experienced significantly lower worker weights and slower foraging rates (Morandin et al. 2005). The neotropical brown stink bug *Euschistus heros* (Hemiptera: Pentatomidae) showed initial positive effects after sub-lethal spinosad exposure with reduced pre-mating latency, but this effect was negated after having a significantly lower net reproductive rate (Santos et al. 2018). In contrast, increased fecundity was observed in the beneficial insect *Orius insidiosus* (Hemiptera: Anthocoridae) after sub-lethal spinosad exposure (Elzen 2001). While the results were not

quantified, morphological defects were found in the sperm of the adult red palm weevil *Rhynchophorus ferrugineus* (Coleoptera: Dryophthoridae), the variety of defects rising with each increase in concentration from 10, 50, 100, and 200 ppm of spinosad (Abdelsalam et al. 2016). These studies show repeated observations of negative effects, with one exception, after sub-lethal spinosad exposure. The implications of these results cannot be assumed to be the same for mosquitoes as these studies evaluated different LC values on a variety of insects with drastically different sensitivities, but they do provide general insight into sub-lethal spinosad exposure. Now that this product is labeled for larval mosquito control it is necessary to further evaluate the potential for sub-lethal effects on mosquitoes of public health concern.

### **1.7.2 *Lysinibacillus sphaericus***

*Lysinibacillus sphaericus*, formerly known as and still frequently referred to as *Bacillus sphaericus*, is a spore forming soil bacterium with insecticidal properties (Ahmed et al. 2007). The spores are comprised of two important proteins, BinA and BinB, which are both necessary for toxicity in mosquito larvae (Broadwell et al. 1990). These spores must be ingested by the mosquito larva, after which they are solubilized in the alkaline midgut, producing larvicidal toxins (Yousten 1984, Charles 1987). While the exact mechanism of toxicity is unknown, the toxins are received into the cytosol of the midgut epithelial cells through receptor mediated endocytosis (Lekakarn et al. 2015). It is proposed that the toxins then cause apoptosis through either autophagy or cytotoxicity, both having the potential to lead to sepsis and death (Tangsongcharoen et al. 2017). Not only is *L. sphaericus* particularly effective against *Culex* mosquitoes in laboratory experiments, it persists effectively in polluted, organically rich water sources (the natural and ideal larval environment for *Culex* mosquitoes) (Davidson et al. 1984, Tangsongcharoen et al. 2015).

*Lysinibacillus sphaericus* does not effectively control *Aedes* mosquitoes unless it is synergized with toxins from *Bacillus thuringiensis* (Wirth et al. 2000).

Few studies have investigated the effects of sub-lethal exposure of *L. sphaericus* on any insects, let alone mosquitoes. Surviving *Cx. quinquefasciatus* larvae exposed to an LC<sub>50</sub> of *L. sphaericus* showed delayed pupation and decreased adult longevity (Lacey et al. 1987). A colony of *L. sphaericus* resistant *Cx. quinquefasciatus* developed faster than controls in the absence of *L. sphaericus*, but demonstrated significant population reducing fitness tradeoffs through reduced fecundity and fertility (Rodcharoen and Mulla 1997). An important freshwater invertebrate predator, *Laccotrephes griseus* (Hemiptera: Nepidae), experienced reduced feeding, fecundity, and delayed maturation after exposure to 25 to 200 ppm of *L. sphaericus*, the severity of these effects increasing with each increased dose (Mathavan et al. 1987). However, a three year study in Wisconsin found that *L. sphaericus* treatments had no observable negative effects on populations of 159 different invertebrate taxa, finding only a reduction in mosquito populations (Merritt et al. 2005). *Lysinibacillus sphaericus* has powerful mosquitocidal properties with few demonstrated risks to non-target organisms, but the sub-lethal effects on the target organism, the mosquito, have not been thoroughly investigated.

### **1.7.3 Methoprene**

Methoprene is a pesticide that disrupts the molting process of insects by mimicking juvenile hormone (JH). JH is one of two major hormones that regulate the insect molting process, the other being 20-hydroxyecdysone. JH is biosynthesized in the corpora allata, an insect endocrine gland, and is released directly into the hemolymph (Goodman and Cusson 2012). The presence of JH in the insect hemolymph encourages larval tissue growth and prevents precocious adulthood; the absence of JH in the presence of 20-hydroxyecdysone initiates a pupal commitment and

prevents a larval to larval molt (Jindra et al. 2013). When late instar insect larvae are exposed to the JH analog methoprene, interference occurs in the typical modulation of 20-hydroxyecdysone by JH and the activation of genes related to pupal progression is inhibited (Wu et al. 2006). Specifically, methoprene appears to interrupt the regeneration of midgut tissues from the larval to pupal stage which results in midgut disfigurations and incomplete molting, causing death, in otherwise morphologically typical pupae (Wu et al. 2006). Methoprene is not effective for killing pupal or adult mosquitoes (Staal 1975).

Methoprene has been used to control mosquito populations for decades and many experiments have looked at the sub-lethal effects of methoprene exposure on mosquito larvae and other organisms. A study conducted in Australia found that sub-lethal methoprene exposure differentially affected sexes and species. Sub-lethal methoprene dosed *Aedes vigilax* larvae produced adults that survived half as long as the control group, *Culex annulirostris* females were unaffected while males survived significantly longer than controls, and neither males nor females of *Culex sitiens* were affected in any observed capacity (Ritchie et al. 1997). Exposure to an LC<sub>50</sub> or LC<sub>5</sub> of methoprene negatively affected total egg production, hatch rates, and adult longevity in *Cx. quinquefasciatus* (Robert and Olson 1989). Another experiment evaluating sub-lethal methoprene exposure (0.1 and 0.2 ppb) on *Ae. aegypti* larvae found reduced adult female longevity and glycogen reserves, but no observed effects on adult males (Sawby et al. 1992). A similar experiment with *Ae. aegypti* measured the effects of sub-lethal methoprene exposure (20 ppb) on fecundity, fertility, and longevity and only found a significant reduction in longevity for both males and females (da Silva et al. 2009). *Anopheles dirus* larvae subjected to sub-lethal methoprene exposure (0.1 ppb) showed an increase in male sex ratio (Sithiprasasna et al. 1996).

Most methoprene exposure studies have evaluated standard fitness parameters such as fecundity, fertility, and longevity, but there are other important parameters worth investigating. The following observation was made in a study examining the lethal effects of methoprene on *Ae. aegypti*. Some surviving males failed to successfully complete the genital rotation that occurs in male mosquitoes within 48 hrs post emergence (Spielman and Williams 1966). This observation was followed up with an experiment in which *Ae. aegypti* pupae received topical applications of methoprene. This resulted in a significantly slower rotation of the treatment mosquito genitalia than the controls, but it failed to actually inhibit this rotation and males were still able to mate after the delay (O'Donnell and Klowden 1997). Similar results were observed in *Drosophila melanogaster* (Diptera: Drosophilidae) with some males rendered sterile due to failed genital rotation (Bouchard and Wilson 1987).

The majority of observed effects on mosquitoes after sub-lethal methoprene exposure thus far have been negative, many of them reducing fertility and fecundity, but positive effects have been seen in other insects. If wild pest insects received these benefits the outcomes could be damaging, but in the laboratory they can be redesigned to help control these pests. The sterile insect technique (SIT) utilizes the release of laboratory sterilized males in reducing pest insect populations, with the goal of sterile males sexually outcompeting their wild and fertile counterparts (Klassen and Curtis 2005). *Anastrepha fraterculus* (Diptera: Tephritidae), a pest of economically important horticultural products and a target of the SIT, responds positively to sub-lethal methoprene treatment. Newly emerged adult males receiving a topical application of methoprene were significantly more attractive to, and mated more frequently with, both young and old females for 20 days (Bachmann et al. 2017). At least two genera of this family have economically important pest species that appear to benefit from an early adulthood application of methoprene. Males of

both *Anastrepha* and *Bactrocera* treated with methoprene experienced accelerated sexual maturation and enhanced sexual competition (Teal et al. 2013). These results may augment the success of SIT releases of these pest flies by incorporating early adult methoprene treatments.

Accelerated sexual maturation was also found when adult male cat fleas (Siphonaptera: Pulicidae) were exposed to JH, methoprene and other JH mimics. Male fleas typically need a blood meal before the plug in their epididymis relaxes, which allows sperm to leave the testes (Rothschild et al. 1970). 100% of unfed males exposed to JH or the three mimics showed sperm movement into epididymis after seven days while 0% of control males had any sperm progression without a blood meal (Dean and Meola 1997).

Stored grains are sometimes treated with methoprene to reduce grain pests (Athanassiou et al. 2011). While methoprene treatments do effectively reduce larval populations of grain pests, mature adults actually benefit from these treatments. Adult species of grain pest beetles of the genera *Oryzaephilus*, *Cryptolestes*, and *Tribolium* produce larger volumes of aggregation pheromones after methoprene exposure, leading to higher reproductive rates, negating the initial success of larval control (Pierce et al. 1986). However, this same study suggested that pheromone baited traps could be supplemented with methoprene to boost their efficiency. It is clear that while some insects suffer negative impacts from varying sub-lethal doses of methoprene, some insects benefit from the exposure and these benefits can be manipulated to help control those pests.

## **1.8 Research Objectives**

Mosquitoes facilitate the spread of debilitating and deadly diseases throughout the world. The fitness of adult mosquitoes, including sexual fitness, is dependent upon conditions in the larval habitat, areas intentionally and unintentionally contaminated with pesticides and herbicides. Some effects of these contaminants on mosquito reproductive health are known, but



many are not. There is also scant information about the sperm capacity, in other words the expected amount of sperm at any given age, of *Culex* mosquitoes. By investigating the effects of these common water contaminants on the sperm production of *Cx. quinquefasciatus*, we may be able to better understand the male reproductive process and thereby discover more opportunities to control this worldwide vector. The specific aims of this study are: to quantify baseline sperm production for wild and laboratory reared *Cx. quinquefasciatus* at 1, 4, 7, and 14 dpe; to evaluate the effects of juvenile exposure to sub-lethal concentrations of spinosad, *L. sphaericus*, methoprene, atrazine, and glyphosate on adult sperm production of wild and laboratory reared *Cx. quinquefasciatus*; and to determine whether or not any observed changes from these exposures were due to changes in body size as measured by wing lengths.

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## Chapter 2: *Culex quinquefasciatus* Sperm Counts

### 2.1 Introduction

Mosquitoes transmit zoonotic pathogens by biting infectious animals and subsequently biting susceptible humans. These mosquito borne infections can be asymptomatic, debilitating, or deadly. Malaria, an endemic disease in tropical areas around the world, kills hundreds of thousands of people every year (WHO 2016). The capacity of mosquitoes to vector dangerous, disease causing pathogens provides motivation to control mosquito populations in disease endemic areas. Traditional pesticide use can effectively control some mosquito populations, but studying the biology of mosquitoes provides new and potentially more effective types of control strategies (Lees et al. 2014).

*Culex quinquefasciatus* can vector dangerous pathogens with expensive consequences. The danger this mosquito presents to public health can be measured in both morbidity and dollars. It is an efficient vector of West Nile virus, St. Louis encephalitis virus, and *Wuchereria bancrofti* (Reisen et al. 2005, Bartholomay et al. 2010). Over 40,000 cases of West Nile fever have been identified in the United States since its introduction in 1999 (CDC 2016). A 2005 outbreak of West Nile fever in California cost the county of Sacramento \$2.98 million in direct and indirect costs (Barber et al. 2010). Considering that the United States has periodic West Nile fever outbreaks throughout the range of *Cx. quinquefasciatus*, the costs of this mosquito are not insignificant. While much is known about the female reproductive and gonotrophic cycles of this mosquito, especially as they relate to pathogen transmission, less is known about male reproductive biology.

The quantification and correlation of sperm production with age has only been conducted for three mosquito species: *Aedes aegypti*, *Aedes albopictus*, and *Anopheles arabiensis*. (Ponlawat and Harrington 2007, Helinski and Knols 2009, Hatala et al. 2018). These studies have shown that

male mosquitoes are capable of producing sperm throughout their field-relevant lifetimes and that larval environmental conditions, which influence adult body size, can impact sperm production and mating efficiency (Ponlawat and Harrington 2009, Bader and Williams 2012). These advances in our understanding of the reproductive biology of the male mosquito may provide important baseline information for successful mosquito control programs (Ferguson et al. 2005). Particularly any strategy which includes the release of modified male mosquitoes. There is no current literature on sperm production or capacity for any *Culex* mosquito.

The present study was designed to quantify sperm production of male *Culex quinquefasciatus*. Experiments on laboratory reared insects are used to predict life history traits and fitness of wild insects, but genetic diversity and uncontrollable field conditions affecting wild insects limit possible inferences (Huho et al. 2007). Therefore, these experiments used laboratory reared and wild collected mosquitoes. The first hypothesis of this study was that *Cx. quinquefasciatus* sperm production would increase with age, to a point, for both laboratory and wild mosquitoes. The second hypothesis was that laboratory reared mosquitoes would have lower sperm counts than wild mosquitoes, as was observed in *Ae. aegypti* (Ponlawat and Harrington 2007). These hypotheses were tested by dissecting out the reproductive tracts of laboratory and wild mosquitoes, enumerating sperm subsamples at four different age points, and collecting wing length data (as a proxy for body size) to rule out size based differences.

## **2.2 Materials and Methods**

### **2.2.1 Mosquitoes**

The medical entomology laboratory in the Life Sciences building at Louisiana State University houses an insectary which has a strain of pesticide susceptible *Culex quinquefasciatus* (known as Sebring). The Sebring strain was collected in Sebring, Florida in 1988 and is maintained

by the USDA Agricultural Research Station (Stancil 2000). This colony was provided to the LSU medical entomology laboratory by East Baton Rouge Mosquito Abatement and Rodent Control in January 2015, and has been maintained continuously since that time.

The maintenance of the Sebring colony was as follows. Adult and larval rearing rooms were maintained at 27°C with approximately 80% relative humidity and a 14:10 light:dark cycle. Adult mosquito cages (31 cm<sup>3</sup>) were provided a blood meal of defibrinated chicken blood (Rockland™ Immunochemicals, Limerick, PA) once per week via an artificial membrane system (Hemotek® Ltd, England). Two days after the blood meal, dark cups filled with deionized water were placed inside the adult cages for oviposition. Two to three egg rafts were placed in clear plastic containers holding 1.5 liters of aged, deionized water with 100mg of 1:1 liver powder and brewer's yeast (both from MP Biomedicals LLC, Solon, OH). After hatching, the larvae were fed the same diet, five times per week. Pupae were removed and placed in plastic cups to facilitate eclosion within the adult cages. A 10% sucrose solution was provided in the adult cages ad libitum via saturated dental wicks sitting in 150 ml Erlenmeyer flasks. The sucrose solution was replaced twice a week and the dental wick once a week.

Wild mosquito egg rafts were collected in modified gravid traps. Large plastic buckets (L:1.27 m, W:1.01 m, H:0.45 m) were filled with four liters of water and 50ml of fish fertilizer (Alaska® 5-1-1 Fish Fertilizer) which had infused for two to three days prior to placement. Two traps were placed at time, approximately 30 m apart in an area with known *Cx. quinquefasciatus* activity. The traps were immediately south of a tree-covered residential area and north of the Residential Life Warehouse on the Louisiana State University, Baton Rouge campus (figure 2.1). This location was ideal for collecting *Cx. quinquefasciatus* with its ample tree cover for birds, outdoor dogs, humans, and permanent ditches. The traps were checked for egg rafts one day after

placement. Rafts were carefully extracted with fine mesh fabric, placed into cups of water, and brought back to the insectary. The eggs and larvae were maintained in the same conditions as the Sebring colony. Adults were provided the same sucrose solution ad libitum, but were not blood fed. A wild colony was not established in the laboratory and all experiments using wild mosquitoes used newly collected egg rafts.



**Fig 2.1** Field site for wild *Culex quinquefasciatus* egg raft collection (30.418104, -91.192537).

### 2.2.2 Experimental Design

Six plastic trays containing 1.5 L of aged diH<sub>2</sub>O and 100 mg of larval diet were set up representing six simultaneous replicates, three for the Sebring colony eggs and three for the wild collected eggs. The three Sebring trays each received two egg rafts. All egg rafts came from the same blood feeding event to ensure uniformity in age, heterogeneity in maternal heritage, and that each replicate was a distinct cohort. The three wild trays each received two egg rafts. All egg rafts

were collected at the same time and from the same trap site, again providing age uniformity and maternal heterogeneity. Eggs hatched within 24-48 hrs and were provided 100 mg of larval diet, five days on and two days off.

The following measures were taken to control for larval density. Within 24 hrs after hatching, a new identical tray was assembled for each cohort. Two hundred larvae were collected from their original tray and placed in a new density controlled tray. Remaining larvae from the original trays were discarded. This was repeated for all six cohorts. To date there is not a reliable method for identifying *Culex* eggs to species while maintaining their viability. Once larvae were third instars, 20 larvae per wild tray were taken at random for identification. Morphological characteristics of the wild larvae were evaluated using a dichotomous key (Burkett-Cadena 2013). If any tray was found to contain larvae of any other species, the entire tray was discarded and the experiment was rescheduled until new egg rafts could be collected.

Upon pupation, 80 pupae were removed from each tray using a wide-lipped disposable pipette and placed in small water filled cups. Each cohort's cup of pupae was placed in corresponding and labeled adult cages. A 10% sucrose solution was provided in the adult cages ad libitum via saturated dental wicks sitting in 150 ml Erlenmeyer flasks. At the first signs of eclosion the adult cages were checked at hourly intervals. All eclosed females were aspirated out of the cages and discarded to preserve the virginity of the males. This method is reliable because male mosquitoes are incapable of copulation within the first 24 hrs after eclosion. After a sufficient number of successfully eclosed males were present in each cage (approximately 50), the pupal cups were removed and any remaining pupae were discarded.

Five adult males were removed from each cage at the following time points: 1, 4, 7, and 14 days post-eclosion (dpe). This provided a sample size of 15 mosquitoes per time point, per



population (table 2.1). Mosquitoes were placed in labeled plastic vials and then stored at -20°C until they were dissected.

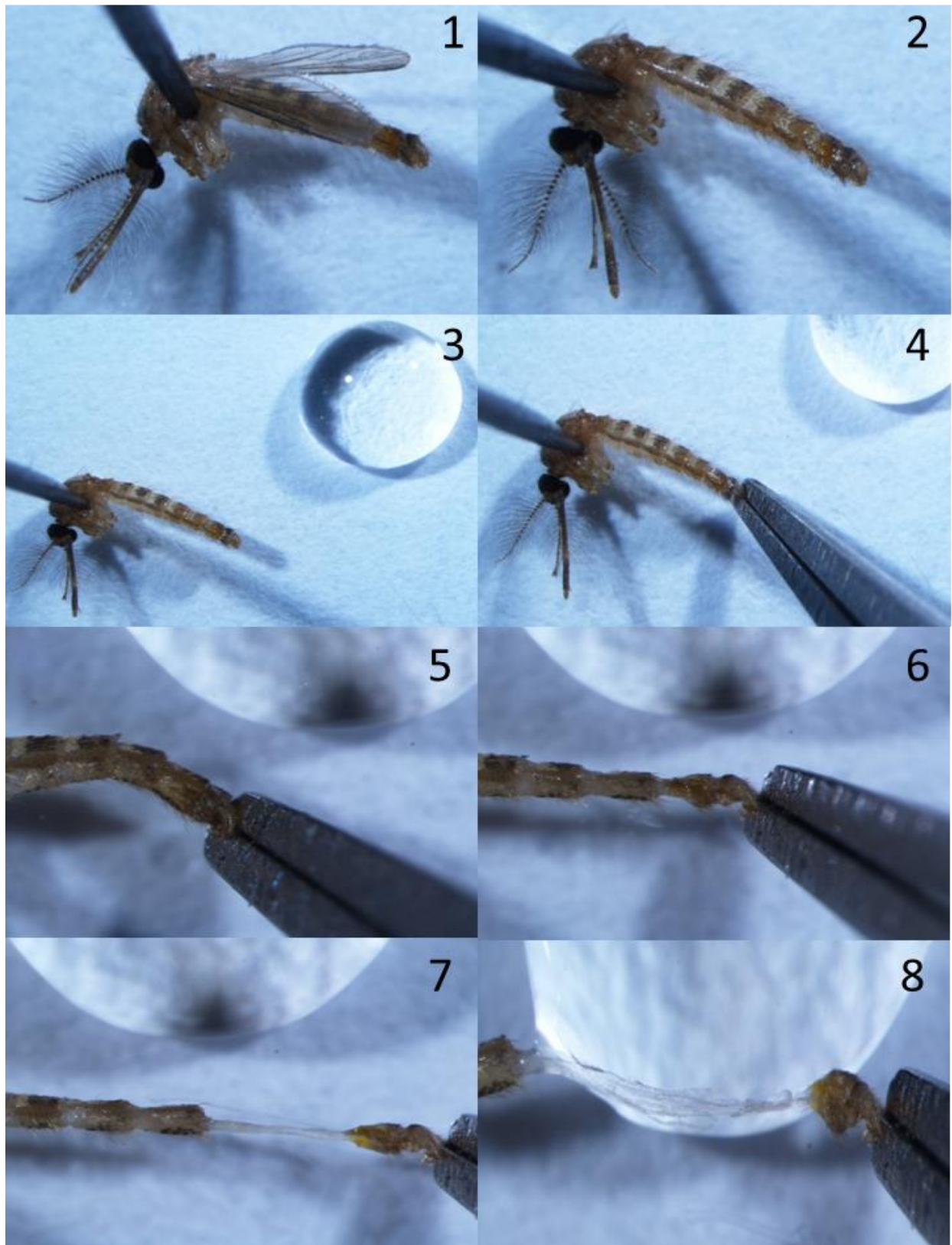
**Table 2.1** Total # of mosquitoes dissected per age and population

Population	1 Day	4 Days	7 Days	14 Days	Total
Sebring	15	15	15	15	60
Wild	15	15	15	15	60

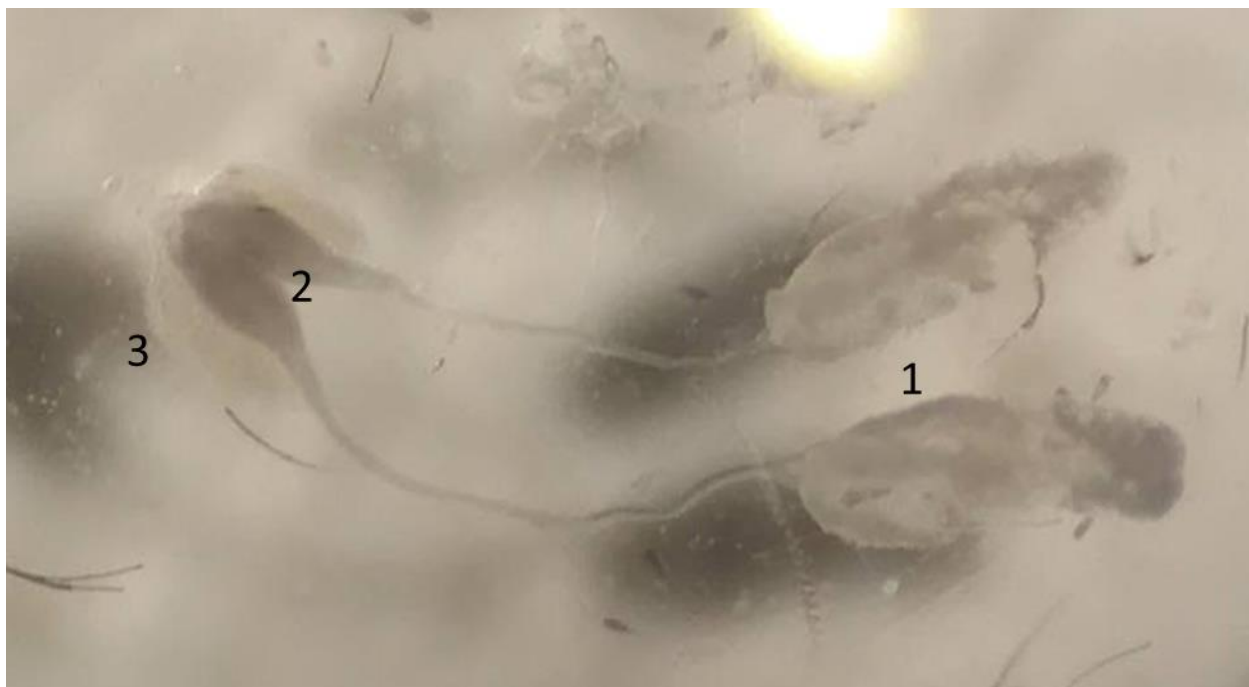
### **2.2.3 Dissection, Quantification, and Wing Lengths**

Each dissection was conducted under a binocular dissecting scope, inside a plastic petri dish, and used the following tools and supplies: fine forceps, an insect pinning needle, a micro needle, deionized water, Wright-Giemsa dye, and microcentrifuge tubes. Successful dissections followed these steps, a modified version of the methods developed by Ponlawat (Ponlawat and Harrington 2007). Mosquitoes were pinned laterally through the thorax and immobilized on the petri dish. Legs were removed and discarded. Wings were removed at their base and the left wing was stored in a 1.5 mL microcentrifuge tube with 70% ethanol. A 10  $\mu$ L drop of deionized water was placed near the distal end of the abdomen. With the mosquito still immobilized via pin through the thorax, the claspers were grabbed using fine forceps. The claspers were pulled gently away from the body until the intersegmental membranes between the last two to three abdominal tergites became visible, translucent, and torn. After the intersegmental membranes were torn, the exposed internal organs were brought into contact with the 10  $\mu$ L drop of water (figure 2.2).

The claspers were then pulled until the testes exited the proximal body cavity. The body was then discarded. Using the insect pin and the micro needle, extraneous internal and external tissues were removed from around the reproductive organs. As this process was likely to remove some water, the isolated reproductive tract, including the testes, accessory glands, and seminal vesicles, was placed in a new 10  $\mu$ L drop of water (figure 2.3).



**Fig 2.2.** Numbered steps of the dissection process for isolating the reproductive tract of male *Culex quinquefasciatus*.



**Fig 2.3.** The male reproductive tract of *Culex quinquefasciatus* showing paired 1. Testes 2. Seminal vesicles 3. Male accessory glands.

Using the pin and the micro needle, these tissues were shredded until no visible pieces remained. This resulted in a slightly clouded final solution. This solution was pipetted into a 1.5 mL microcentrifuge tube, the pin and needle rinsed inside the same tube with 50  $\mu$ L of diH<sub>2</sub>O, and combined with 5  $\mu$ L of solution one and 15  $\mu$ L of solution two of the three step Wright-Giemsa stain, and 120  $\mu$ L of diH<sub>2</sub>O resulting in a final volume of 200  $\mu$ L of dyed sperm solution per mosquito. Each microcentrifuge tube was vortexed individually for 10-20 seconds, and then stirred using a P20 pipette. Once thoroughly mixed, 10 individual 5  $\mu$ L drops were placed on a clean microscope slide and allowed to dry, displaying 50  $\mu$ L, representing one fourth of the total volume of sperm solution for each dissected mosquito. Each drop on each slide was viewed at 40x and the total sperm per drop was counted and recorded (figure 2.4). The left wing was collected and preserved from each successfully dissected mosquito. These wings were measured using a Nikon binocular camera attachment. Lengths were taken from the alular notch to the second radial branch, excluding scales (Bourguet et al. 2004).



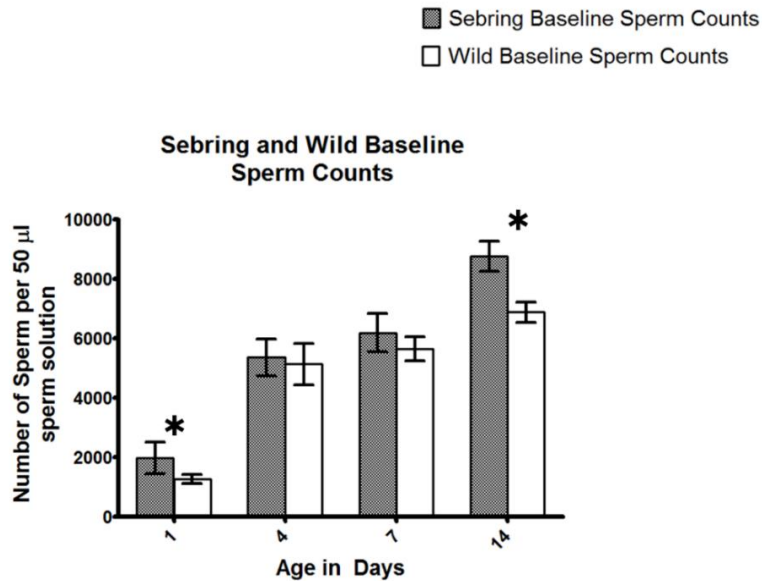
**Fig 2.4.** A labeled slide containing 10 drops of 5  $\mu$ L stained sperm solution, representing 25% of the total sperm solution of one mosquito.

### 2.2.5 Statistical Analysis

Data were analyzed using GraphPad Prism 5© (La Jolla, CA). To accommodate for non-normal distributions and small sample sizes ( $N = 15$  per population per time point), sperm data were subjected to the nonparametric Mann-Whitney U test. Independent variables included population and age, the dependent variable was sperm count. Wing length data were normally distributed and had a large sample size ( $N = 60$  per population) and were analyzed with the Student's t-test. The independent variable was population and the dependent variable was wing length. To determine whether average body size per population influenced sperm count, sperm and wing measurements were combined into one variable, number of sperm/mm of wing length. These data were also subjected to the nonparametric Mann-Whitney U test.

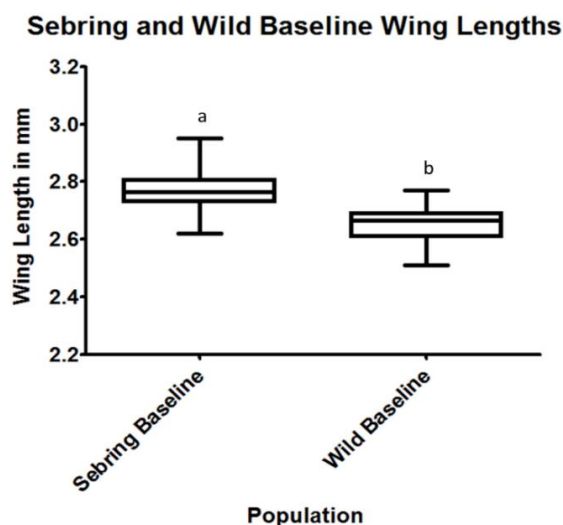
### 2.3 Results

Mann-Whitney U Tests compared the sperm counts of the Sebring population at 1, 4, 7, and 14 dpe to the corresponding wild sperm counts at those ages. Significant differences were found at ages 1 and 14 dpe ( $U = 22$ ,  $P = 0.0002$  and  $U = 0$ ,  $P = <0.0001$ ,  $\alpha = 0.05$ ) when comparing same-aged Sebring and wild mosquitoes (figure 2.5).



**Fig 2.5.** Average number of sperm per 50 µl of sperm solution for Sebring and wild collected *Culex quinquefasciatus* by age in days (N = 120). Paired bars marked by asterisks are statistically significant from each other with  $\alpha = 0.05$ .

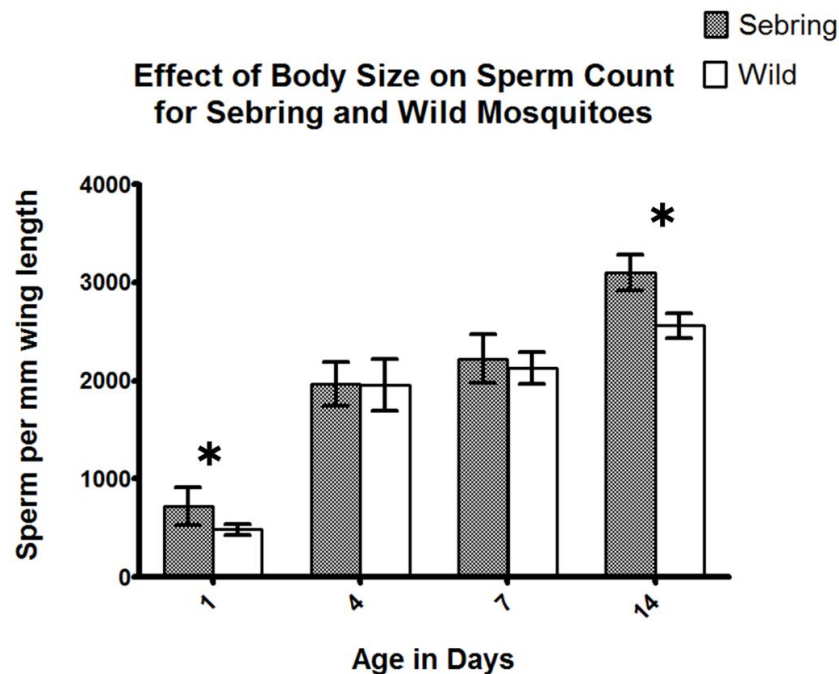
The Student's t-test was used to analyze wing length data from both populations. Adult mosquitoes do not grow over time, so dpe was not factored into the analysis. Therefore, all wing lengths per population were analyzed together regardless of age at the time of dissection. Sebring mosquitoes had significantly larger wings ( $P = <0.0001$ ,  $N = 60$ ,  $t = 9.989$ ,  $df = 118$ ,  $\alpha = 0.05$ ) than wild mosquitoes. Means and range of wing length distribution are shown (figure 2.6).



**Fig 2.6.** Average wing length for Sebring and wild collected *Culex quinquefasciatus* (N = 120). Plots with different letters are statistically significant with  $\alpha = 0.05$ .

Adult body size has been positively correlated with sperm production in some mosquitoes (Ponlawat and Harrington 2007). To determine whether the difference in average body size between the Sebring and wild mosquitoes was affecting sperm count, sperm and wing length measurements were reduced to one variable, number of sperm/mm of wing length.

After adjusting these variables to account for body size bias, no change in statistical significance was observed. Sperm counts at 1 and 14 dpe were still significant ( $U = 25$ ,  $P = 0.0003$  and  $U = 0$ ,  $P = <0.0001$ ,  $N = 15$ ,  $\alpha = 0.05$ ), sperm counts at 4 and 7 were not (figure 2.7).



**Fig 2.7.** Average number of sperm per/mm wing length for Sebring and wild collected *Culex quinquefasciatus* by age in days ( $N = 120$ ). Paired bars marked by asterisks are statistically significant from each other with  $\alpha = 0.05$ .

## 2.4 Discussion

The first hypothesis of this study was that *Culex quinquefasciatus* sperm production would increase with age for both laboratory and wild mosquitoes and analysis of these data supports this hypothesis. Both the Sebring and wild populations showed continued increases in sperm count from 1 to 14 dpe. While these data did not show any senescence related decreases in sperm, it is

expected that data collected beyond this time range would show a plateau effect and an eventual decrease in sperm production with age (Ponlawat and Harrington 2007). These data also show that older virgin males have greater reproductive capacity than younger virgin males. Radio-sterilization of adult male mosquitoes produced the most competitive males when compared to males sterilized as pupae (Andreasen and Curtis 2005), and 5-day-old sterile males significantly outcompeted 1-day-old sterile males (Oliva et al. 2012). The current study supports these findings. For example, the mean sperm count for Sebring day 4 males was 5,373 vs the Sebring day 14 males at 8,772 (and these numbers only represent 25% of the total sperm per mosquito). The difference of 10 days shows a 61.25% increase in sperm of these *Cx. quinquefasciatus*. MAG size also increases with age, a known limiting factor of insemination ability for male mosquitoes (Foster and Lea 1975). With increased sperm counts and larger MAG substance capacity it is not unreasonable to hypothesize that older, virgin males would be able to inseminate more females than their younger counterparts.

The second hypothesis of this study was that laboratory reared mosquitoes would have lower sperm counts than wild mosquitoes, but the analysis of these data does not support this hypothesis. The Sebring colony had significantly higher sperm counts at 1 and 14 dpe, and numerically higher sperm counts at 4 and 7 dpe (no statistical significance). To date, only one study has compared sperm counts of laboratory and wild mosquitoes. The results of that study showed higher sperm counts in wild *Ae. aegypti* than in two different laboratory colonies of the same species (Ponlawat and Harrington 2007). Three potential explanations for this apparent contradiction (beyond the simple fact that these are different species with different life histories and reproductive strategies) are presented.



First, the wild *Ae. aegypti* in the Ponlawat and Harrington study were collected as pupae and separated by sex after eclosion. This means that almost the entire developmental period of these mosquitoes was governed by uncontrolled variables, of particular note, the larval diet. Larval diet not only impacts adult longevity and growth, but reproductive maturation and fecundity as well (Farkas and Brust 1985, Joy et al. 2010). This suggests that the *Ae. aegypti* collected in Ponlawat and Harrington's study may have come from a larval environment with a more nutrient rich diet than what was supplied to the larvae in their laboratory colony. In the present study, wild eggs were collected and hatched in laboratory conditions. Wild and Sebring larvae were reared on identical diets and in identical environments, eliminating diet and environmental fluctuations as possible factors in sperm production in this study.

Second, Boggs explains that resource allocation is not a static function in organisms, but is dynamically dependent upon external stimuli, especially nutrient availability. Adult mosquito nutrient acquisition and allocation can be modeled after a triangle. At the top, nutrient acquisition occurs, and depending upon the needs of the mosquito in its current environment and physiological state, nutrients are allocated. Nutrients down one leg of the triangle go to reproduction energy, down the other to survival energy, or from one leg to the other at the bottom if needs have changed after allocation has already occurred. This type of allocation necessitates tradeoffs, e.g. flight energy may decrease during oogenesis (Boggs 2009). A well-established example of this process is in the adult female mosquito. In adverse conditions, sugar questing will be prioritized and nutrients will be used to ensure survival; in ideal conditions, blood meal questing will be prioritized to support reproduction (Foster 1995). If a female mosquito begins oogenesis, but is then found in adverse conditions, she may resorb nutrients from the ovarian follicles to increase her chances of survival (Clifton and Noriega 2011). Similar conclusions may be drawn for adult male mosquito



nutrient allocation. Males require sugar meals to sustain flight energy, up to 50% of which is used during pre-mating swarming behavior (Yuval et al. 1994). Sugar meals also fuel sperm production and insemination which occur at significant energetic costs (Bargielowski et al. 2011). Insemination costs have been shown to be taxing enough to reduce longevity by up to 15 days (South et al. 2009). When comparing the relative stress of laboratory mosquitoes (abundant food, limited flight space, and a complete lack of predators) to that of wild mosquitoes (flight energy spent on finding food, mating partners, and avoiding predators) in the context of the triangle model of nutrient allocation, higher sperm counts in laboratory reared males would be expected. Evidence supporting this idea was found when multi-generational laboratory reared males had significantly lower nutrient reserves than wild mosquitoes collected and reared in laboratory conditions, reflecting the prioritization of nutrient allocation for survival (Huho et al. 2007).

Third, the artificial selection pressure on laboratory colony insects induces genetic and behavioral changes (Mason et al. 1987). The olive fruit fly *Dacus oleae* (Diptera: Tephritidae) experienced genetic changes at three observed loci after only four generations of laboratory colony conditions (Loukas et al. 1985). Observed behavioral changes in multi-generational laboratory colonies include reduced dispersal when released, reduced mating compatibility with wild conspecifics, and reduced mate finding (Boller 1972). Laboratory reared *Anopheles quadrimaculatus* intended for SIT release were unable to locate females in the wild, a failure attributed to behavioral changes from colony selection pressure (Dame et al. 1964). Another study showed that *An. quadrimaculatus* reared in the laboratory had significantly lower flight capacity which, again, led to failed SIT releases (Rai 1969). The most significant effect of laboratory induced changes supporting the observations of this study were found in a laboratory colony of *An. arabiensis*. Colonization pressure on these mosquitoes hastened sexual maturation in males,

demonstrated by 96% of males having completed their genital rotation by 17 hrs post-eclosion (compared to 0% of wild males) and successful copulation by some colony males as early as 11 hrs post-eclosion (Oliva et al. 2011). The timing of genital rotation was not observed in the present study, but the significantly higher sperm production in the Sebring colony at 1 dpe suggests accelerated sexual maturation, possibly induced by artificial selection pressure in the laboratory.

The Sebring mosquitoes had significantly larger wing lengths than the wild mosquitoes. When larval conditions are consistent, as they were in this study, larger males are expected to have higher sperm counts (Ponlawat and Harrington 2007, Bader and Williams 2012). To determine whether or not the observed difference in body size was a determining factor in sperm production, the two dependent variables (number of sperm and wing length) were combined into one variable, number of sperm/mm of wing length. After analyzing this new variable by 1, 4, 7, and 14 dpe, it was determined that the numerically slight, but statistically significant difference in wing length did not affect the sperm count of these mosquitoes. It is, therefore, more reasonable to suggest that one or more of the proposed explanations above are responsible for the difference between the Sebring and wild sperm counts.

This is the first recorded study quantifying the spermatozoa of *Cx. quinquefasciatus* through actual enumeration rather than prediction based on spermatocyst number (Jones 1967). As a globally distributed species capable of vectoring dangerous pathogens, fundamental advances in our understanding of the reproductive biology of both sexes is important. This study suggests that male *Cx. quinquefasciatus* sperm production increases over time. This study also suggests that Sebring colony mosquitoes produce more sperm than locally collected wild conspecifics. While the exact cause of this difference is unknown, potential explanations include different priorities for nutrient allocation, or artificial selection pressure induced by colonization. These results serve as

good reminder that care should be taken when laboratory colony collected data is used to infer qualities or potential of wild populations of mosquitoes. Future research looking at sperm production of different populations of wild and laboratory mosquitoes would help clarify these points.

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## Chapter 3: Effects of Pesticide Exposure on Sperm Production

### 3.1 Introduction

Controlling mosquito populations is an important part of reducing the incidence of mosquito borne diseases (Carney et al. 2008). Traditional pesticide use can effectively control some mosquito populations, but studying the biology of mosquitoes provides new and potentially more effective types of control strategies. Because the reproductive system is a potential target for control strategies, the female reproductive and gonotrophic cycles of known vector species from the genera *Aedes*, *Anopheles*, and *Culex* have been well studied (Stouthamer et al. 1999, Briegel and Timmermann 2001, Zhu and Noriega 2016). Far less is known about the male reproductive system of these mosquitoes, particularly regarding sperm production.

The effects of irradiation and chemical exposure on mosquito sperm production have only been evaluated for one species of mosquito, *Anopheles arabiensis*. The two studies evaluating these effects did so with a focus on the effects of radio-sterilization of mosquitoes for SIT releases. The first study irradiated either pupae or 6-day-old adults with 70 or 120 Gy and found that pupal, but not adult, irradiation at both doses significantly decreased sperm production (Bader and Williams 2012). The second study exposed pupae to 70 Gy, the organochloride dieldrin, or both. All three treatments showed an initial sperm reduction at 2 days post eclosion (dpe), but the only treatment that did not recover, recovery defined by increased sperm production, by 6 dpe was the 70 Gy treated group (Damiens et al. 2013). These experiments demonstrated the effects of sterilization on the sperm production of *Anopheles* mosquitoes during the pupal and adult stages.

The majority of spermatogenesis occurs during the larval stage, providing the largest window of opportunity for chemical disruption of sperm production (Horsfall and Ronquillo 1970, Dumser 1980). The studies by Bader and Damiens show that irradiation and chemical stressors

can indeed effect sperm production in mosquitoes, but these studies only exposed pupae to these compounds. It is not known if sub-lethal chemical concentrations, such as residual larvicides or herbicides, in the habitats of larval mosquitoes have any effect on adult sperm production. Understanding how chemical exposure effects sperm production in wild mosquitoes could help us better understand the reproductive biology of wild mosquitoes.

The present study was designed to examine the effects of larval exposure to sub-lethal concentrations of pesticides and herbicides on the sperm production of adult *Cx. quinquefasciatus*. Experiments on laboratory reared insects are used to predict life history traits and fitness of wild insects, but genetic diversity and uncontrollable field conditions effecting wild insects limit possible extrapolations (Huho et al. 2007). Therefore, these experiments used laboratory reared and wild collected mosquitoes. The first hypothesis of this study was that sperm production of wild collected and laboratory reared mosquitoes would be negatively affected by larval exposure to sub-lethal concentrations of spinosad, *Lysinibacillus sphaericus*, methoprene, atrazine, and glyphosate. The second hypothesis of this study was that these chemical exposures would not affect body size, measured by wing length, so that any observed reduction in sperm would be caused by mechanisms other than retarded somatic growth. These hypotheses were tested by exposing third instar larvae to one of the five pesticides, dissecting out the adult reproductive tracts, conducting sperm counts at four age points, and collecting wing length data.

## **3.2 Materials and Methods**

### **3.2.1 Mosquitoes**

The medical entomology laboratory in the Life Sciences building at Louisiana State University houses an insectary which has a strain of pesticide susceptible *Culex quinquefasciatus* (known as Sebring). The Sebring strain was collected in Sebring, Florida in 1988 and is maintained

by the USDA Agricultural Research Station (Stancil 2000). This colony was provided to the LSU medical entomology laboratory by East Baton Rouge Mosquito Abatement and Rodent Control in January 2015, and has been maintained continuously since that time.

The maintenance of the Sebring colony was as follows. Adult and larval rearing rooms were maintained at 27°C with approximately 80% relative humidity and a 14:10 light:dark cycle. Adult mosquito cages (31 cm<sup>3</sup>) were provided a blood meal of defibrinated chicken blood (Rockland™ Immunochemicals, Limerick, PA) once per week via an artificial membrane system (Hemotek® Ltd, England). Two days after the blood meal, dark cups filled with deionized water were placed inside the adult cages for oviposition. Two to three egg rafts were placed in clear plastic containers holding 1.5 L of aged, deionized water with 100 mg of 1:1 liver powder and brewer's yeast (both from MP Biomedicals LLC, Solon, OH). After hatching, the larvae were fed the same diet, five times per week. Pupae were removed and placed in plastic cups to facilitate eclosion within the adult cages. A 10% sucrose solution was provided in the adult cages ad libitum via saturated dental wicks sitting in 150 ml Erlenmeyer flasks. The sucrose solution was replaced twice a week and the dental wick once a week.

Wild mosquito egg rafts were collected in modified gravid traps. Large plastic buckets (L:1.27 m, W:1.01 m, H:0.45 m) were filled with four liters of water and 50ml of fish fertilizer (Alaska® 5-1-1 Fish Fertilizer) which had infused for two to three days prior to placement. Two traps were placed at a time, approximately 30 m. apart in an area with known *Cx. quinquefasciatus* activity. The traps were immediately south of a tree covered residential area and north of the Residential Life Warehouse on the Louisiana State University, Baton Rouge campus. This location was ideal for collecting *Cx. quinquefasciatus* with its ample tree cover for birds, outdoor dogs, humans, and permanent ditches. The traps were checked for egg rafts one day after placement.



Rafts were carefully extracted with fine mesh fabric, placed into cups of water, and brought back to the insectary. The eggs and larvae were maintained in the same conditions as the Sebring colony. Adults were provided the same sugar water solution ad libitum, but were not blood fed. A wild colony was not established in the laboratory and all experiments using wild mosquitoes used newly collected egg rafts.

### **3.2.2 Experimental Design**

Twelve plastic trays, each containing 1.5 L of aged diH<sub>2</sub>O and 100mg of larval diet (1:1 liver powder and brewer's yeast), were set up. Six trays were used for Sebring colony experiments and six were used for wild collected mosquitoes. Three trays per population were treated with a pesticide and the remaining three were controls. The six Sebring trays each received two egg rafts. All egg rafts came from the same blood feeding event to ensure uniformity in age, heterogeneity in maternal heritage, and that each replicate was a distinct cohort. The six wild trays each received two egg rafts. Egg rafts were collected at the same time and from the same trap site, again providing age uniformity and maternal heterogeneity. Wild eggs were not established as a colony, but were collected from the wild at the beginning of each experiment. Eggs hatched within 24-48 hrs and were provided 100mg of larval diet, five days on and two days off. This design was repeated five times, once for each of the following pesticide or herbicide treatments: spinosad, *L. sphaericus*, methoprene, atrazine, and glyphosate.

The following measures were taken to control for larval density. Within 24 hrs after hatching, a new identical tray was assembled for each cohort. Approximately 200 larvae were collected from their original tray and placed in a new density controlled tray. Remaining larvae from the original trays were discarded. This was repeated for all six treatments and all six controls, per each chemical treatment. To date there is not a reliable method for identifying wild *Culex* eggs

to species while maintaining their viability. Once larvae were third instars, 20 larvae per wild tray were taken at random for identification. Morphological characteristics of the wild larvae were evaluated using a dichotomous key (Burkett-Cadena 2013). If any tray was found to contain larvae of any other species the entire tray was discarded and the experiment was rescheduled until new egg rafts could be collected.

Once larvae from both populations were third instars, sub-lethal concentrations of pesticide were added to each treatment tray (table 3.1). The spinosad, *L. sphaericus*, and methoprene treatments received laboratory determined LC<sub>10</sub> doses and the atrazine and glyphosate treatments received field relevant doses as determined by field collected water contamination data (Gilliom and Hamilton 2006, Bara et al. 2014). Technical grade atrazine was dissolved in ethanol, technical grade glyphosate was dissolved in chloroform, technical grade spinosad was dissolved in acetone, and the water soluble formulated products *L. sphaericus* and methoprene were not dissolved.

**Table 3.1:** Sub-lethal concentrations of active ingredient used in sperm quantification assays.

Product	PPM of Active Ingredient
Spinosad	0.007
<i>B. sphaericus</i>	0.000028
Methoprene	0.0003
Atrazine	5
Glyphosate	5

Eighty pupae were removed from each tray using a wide-lipped disposable pipette and placed in small water filled cups. Each cohort's cup of pupae was placed in corresponding and labeled adult cages. A 10% sucrose solution was provided in the adult cages ad libitum via saturated dental wicks sitting in 150 ml Erlenmeyer flasks. At the first signs of eclosion the adult cages were checked at hourly intervals. All eclosed females were aspirated out of the cages and discarded to preserve the virginity of the males. This method is reliable because male mosquitoes are incapable of copulation within the first 24 hrs after eclosion. After a sufficient number of

successfully eclosed males were present in each cage (approximately 50), the pupal cups were removed and any remaining pupae were discarded.

Five adult males were removed from each treatment cage and two adult males from each control cage at the following time points: 1, 4, 7, and 14 dpe. This provided a sample size of 15 treatment and 6 control mosquitoes per time point, per population, per chemical treatment (tables 3.2 and 3.3). Removed males were kept in labeled plastic vials and stored at -20°C until dissection

**Table 3.2.** Total number of Sebring mosquitoes dissected after pesticide treatments.

	Sebring	1 Day	4 Days	7 Days	14 Days	Total
Treatment	Spinosad	15	15	15	15	60
	<i>L. sphaericus</i>	15	15	15	15	60
	Methoprene	15	15	15	15	60
	Atrazine	15	15	15	15	60
	Glyphosate	15	15	15	15	60
Control	Spinosad	6	6	6	6	24
	<i>L. sphaericus</i>	6	6	6	6	24
	Methoprene	6	6	6	6	24
	Atrazine	6	6	6	6	24
	Glyphosate	6	6	6	6	24
	Total	105	105	105	105	420

**Table 3.3.** Total number of wild mosquitoes dissected after pesticide treatments.

	Wild	1 Day	4 Days	7 Days	14 Days	Total
Treatment	Spinosad	15	15	15	15	60
	<i>L. sphaericus</i>	15	15	15	15	60
	Methoprene	15	15	15	15	60
	Atrazine	15	15	15	15	60
	Glyphosate	15	15	15	15	60
Control	Spinosad	6	6	6	6	24
	<i>L. sphaericus</i>	6	6	6	6	24
	Methoprene	6	6	6	6	24
	Atrazine	6	6	6	6	24
	Glyphosate	6	6	6	6	24
	Total	105	105	105	105	420

### **3.2.3 Pesticide Concentration Determination**

The field relevant, sub-lethal concentration of herbicides, 5ppm of atrazine and glyphosate, were determined by evaluating available literature on surface water contamination of these products (Gilliom and Hamilton 2006, Bara et al. 2014). The LC<sub>10</sub> of spinosad and *Lysinibacillus sphaericus* were determined by interpreting data previously collected from the same Sebring colony, by Nick DeLisi, the author having helped set up and collect data from these experiments (DeLisi 2017).

A diagnostic dose response curve for methoprene was developed by exposing third instar larvae of the Sebring colony to five different concentrations formulated Methoprene, ~20 larvae per glass cup with three replicates at each concentration. This experiment was conducted five times on five different dates. Methoprene induced mortality is measured in holometabolous insects by observing the number of unsuccessfully vs successfully eclosed adults post treatment. The following formula has been developed to calculate percent inhibited eclosion (%IE), a percent that can be used to represent mortality.  $\%IE = 100 - 100(T/C)$ , where T = % of successfully eclosed treatment subjects and C = % of successfully eclosed control subjects (WHO 2005). The %IE values were subjected to probit analysis to determine LC<sub>50</sub> and LC<sub>10</sub> values (0.0035 and 0.0003, respectively) using StatGraphics18© (Warrenton, VA).

### **3.2.4 Dissection, Quantification, and Wing Lengths**

Each dissection was conducted under a binocular dissecting scope, inside a plastic petri dish, and used the following tools and supplies: fine forceps, an insect pinning needle, a micro needle, deionized water, Wright-Giemsa dye, and microcentrifuge tubes. Dissections followed these steps, a modified version of the methods developed by Ponlawat (Ponlawat and Harrington 2007). Mosquitoes were pinned laterally through the thorax on the petri dish. Legs were removed

and discarded. Wings were removed at their base and the left wing was stored in a 1.5 mL microcentrifuge tube with 70% ethanol. A 10  $\mu$ L drop of diH<sub>2</sub>O was placed at the distal end of the abdomen. With the mosquito still immobilized via pin through the thorax, the claspers were grabbed using fine forceps. The claspers were pulled gently away from the body until the intersegmental membranes between the last two to three abdominal tergites became visible, translucent, and torn. The exposed internal organs were then brought into contact with the 10  $\mu$ L drop of water. The claspers were then pulled until the testes exited the proximal body cavity. The body was then discarded. Using the insect pin and the micro needle, extraneous internal and external tissues were removed from around the reproductive organs. As this process was likely to remove some water, the isolated reproductive tract, including the testes, accessory glands, and seminal vesicles, was placed in a new 10  $\mu$ L drop of water. Using the pin and the micro needle, all of the present tissues were shredded until no identifiable tissues remained, resulting in a slightly clouded final solution. This solution was pipetted into a 1.5 mL microcentrifuge tube, the pin and needle rinsed inside the same tube with 50  $\mu$ L of diH<sub>2</sub>O, and combined with 5  $\mu$ L of solution one and 15  $\mu$ L of solution two of the three step Wright-Giemsa stain, and 120  $\mu$ L of diH<sub>2</sub>O resulting in a final volume of 200  $\mu$ L of dyed sperm solution per mosquito. Each microcentrifuge tube was vortexed individually for 10-20 seconds and then stirred using a P20 pipette. Once thoroughly mixed, 10 individual 5  $\mu$ L drops were placed on a clean microscope slide and allowed to dry, displaying 50  $\mu$ L, representing one fourth of the total volume of sperm solution for each dissected mosquito. Each drop on each slide was viewed at 40x and the total sperm per drop was counted and recorded. The left wing was collected and preserved from each successfully dissected mosquito. These wings were measured using a Nikon binocular camera attachment. Lengths were taken from the alular notch to the second radial branch, excluding scales (Bourguet et al. 2004).

### 3.2.5 Statistical Analysis

Both sperm count and wing length data were analyzed using GraphPad Prism 5© (La Jolla, CA). To accommodate for non-normal distributions and small sample sizes (N = 15 per population, per time point, per treatment; control N = 6 per population, per time point, per treatment), sperm data were subjected to the nonparametric Mann-Whitney U test. Separate statistical comparisons were made between the treatment and corresponding control group for each chemical (i.e., statistical comparisons were not made among chemical treatments or mosquito populations. Wing length data (N = 60 per population, per treatment; control N = 24 per population, per treatment) and were analyzed with the nonparametric Kruskal-Wallis one-way ANOVA with the post hoc Dunn's test.

### 3.3 Results

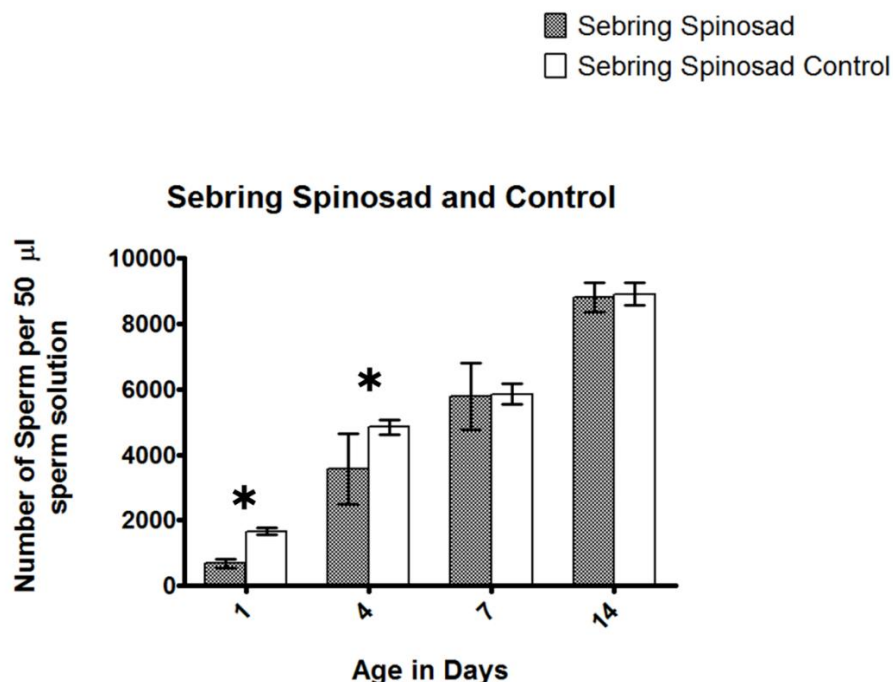
Spinosad treatments of the Sebring colony larvae showed a significant decrease in sperm at 1 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05) and 4 dpe (U = 9, P = 0.0057,  $\alpha$  = 0.05), high variation at 4 and 7 dpe, and no difference in sperm count or variation at 14 dpe (figure 3.1).

*Lysinibacillus sphaericus* treatments of the Sebring colony larvae showed a significant decrease in sperm at 1 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05) and no difference in sperm count or variation at 4, 7, or 14 dpe (figure 3.2).

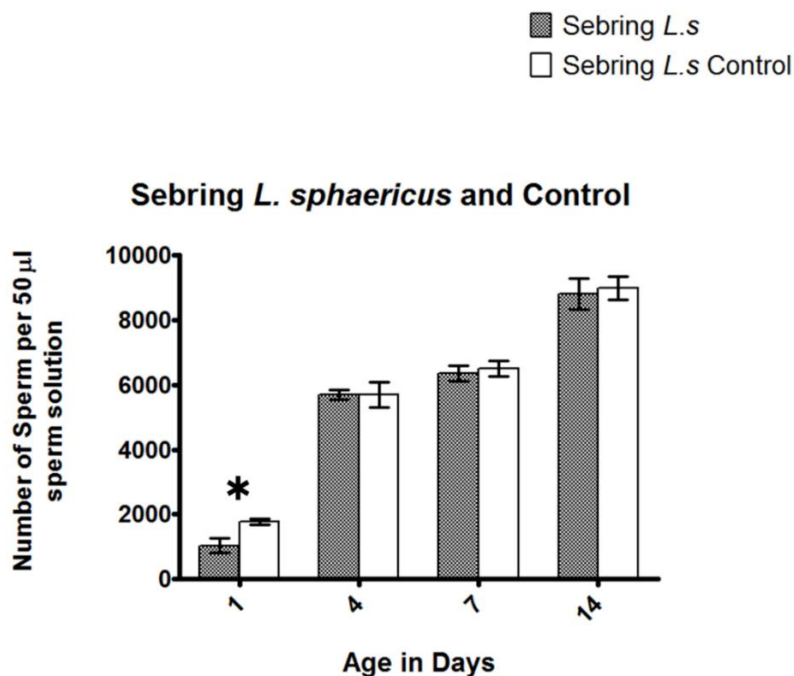
Methoprene treatments of the Sebring colony larvae showed a significant increase in sperm at 1 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05), 4 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05), and 7 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05) with no difference in sperm count at 14 dpe (figure 3.3).

Atrazine treatments of the Sebring colony larvae showed no significant difference in sperm count at any age point (figure 3.4).

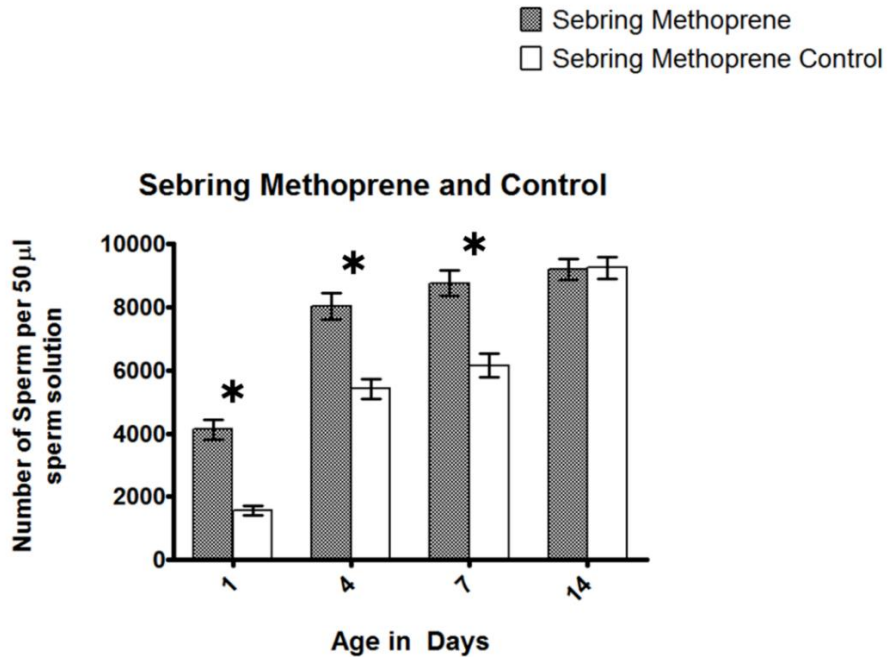
Glyphosate treatments of the Sebring colony larvae showed a significant decrease in sperm at 4 dpe (U = 0, P = 0.0005,  $\alpha$  = 0.05), but no difference at 1, 7, or 14 dpe (figure 3.5).



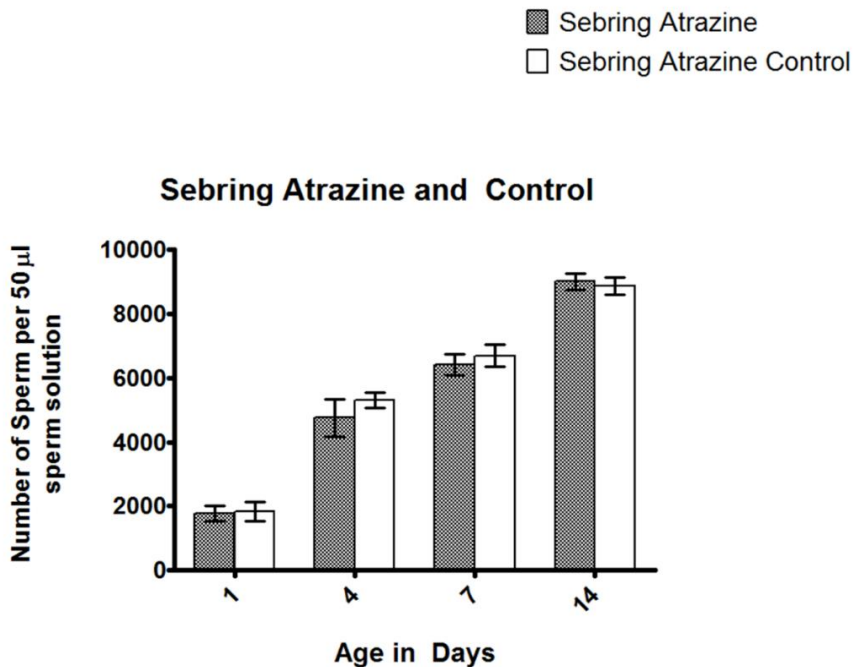
**Fig 3.1.** Average number of sperm per 50µl of sperm solution for Sebring *Culex quinquefasciatus* treated with spinosad by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .



**Fig 3.2.** Average number of sperm per 50µl of sperm solution for Sebring *Culex quinquefasciatus* treated with *Lysinibacillus sphaericus* by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .

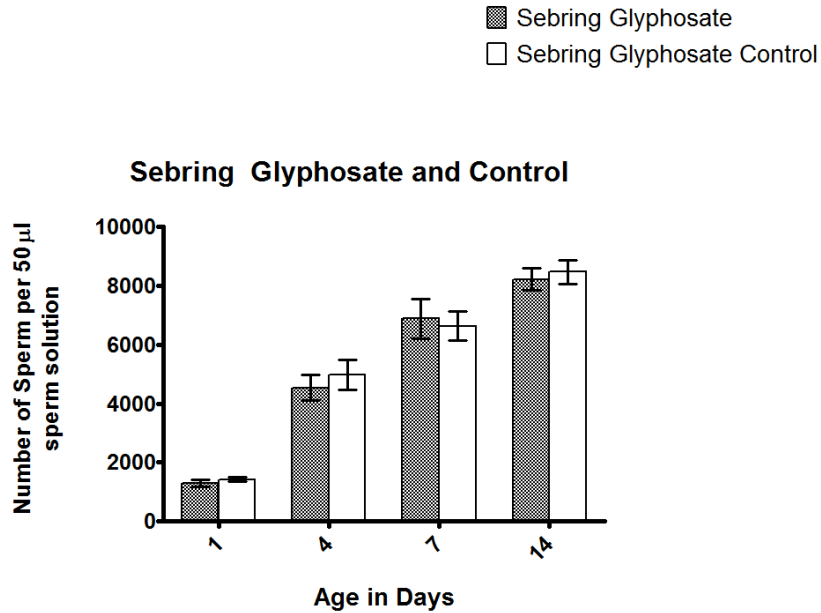


**Fig 3.3.** Average number of sperm per 50µl of sperm solution for Sebring *Culex quinquefasciatus* treated with methoprene by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .



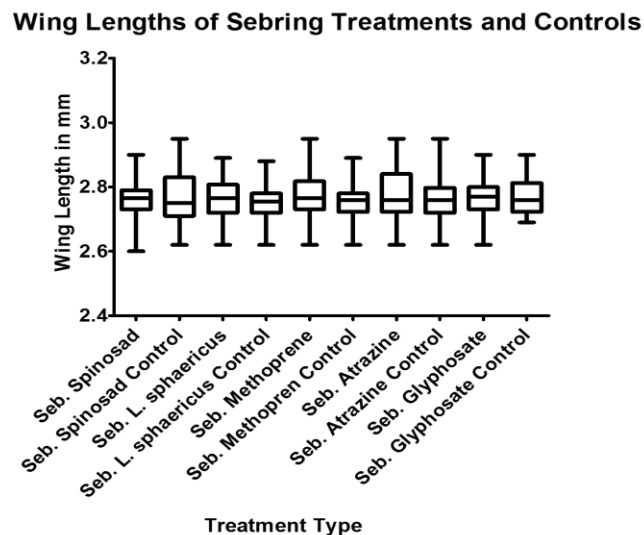
**Fig 3.4.** Average number of sperm per 50µl of sperm solution for Sebring *Culex quinquefasciatus* treated with atrazine by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .





**Fig 3.5.** Average number of sperm per 50µl of sperm solution for Sebring *Culex quinquefasciatus* treated with glyphosate by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .

The nonparametric Kruskal-Wallis one-way ANOVA was used to determine differences in wing length among Sebring treatments and controls. There was no overall statistical significance, nor any specific differences after using the post hoc Dunn's test to compare each individual treatment to its control (figure 3.6).



**Fig 3.6.** Average wing length for Sebring *Culex quinquefasciatus* (N = 420). Plots with different letters are statistically significant with  $\alpha = 0.05$ .

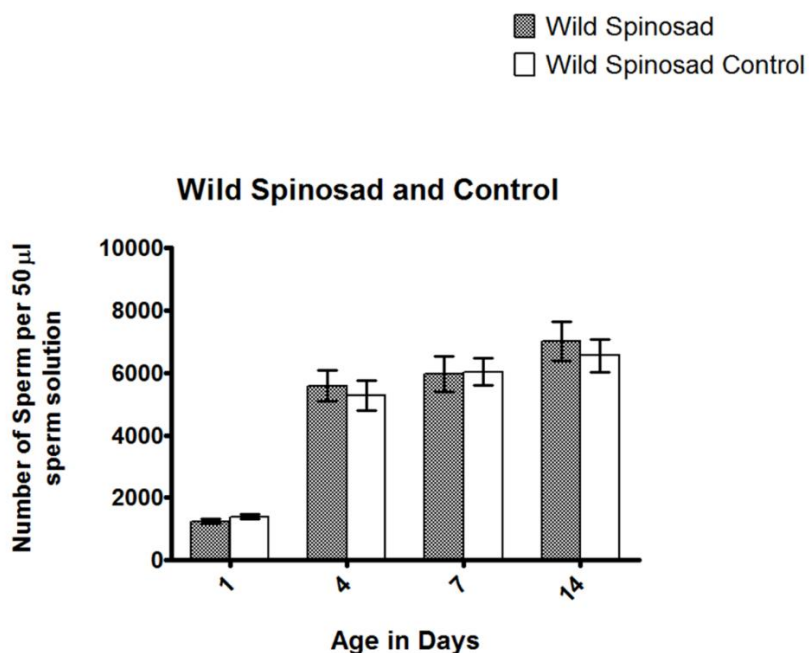
Spinosad treatments of the wild larvae showed no significant difference in sperm count at any age point (figure 3.7).

*Lysinibacillus sphaericus* treatments of the wild larvae showed no significant difference in sperm count at any age point (figure 3.8).

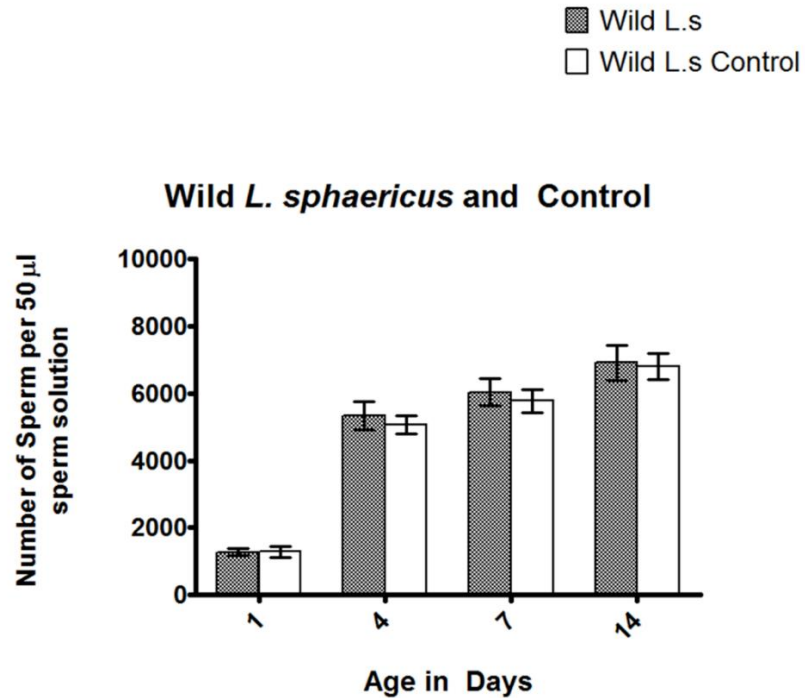
Methoprene treatments of the wild larvae showed a significant increase in sperm at 1 dpe ( $U = 0$ ,  $P = 0.0005$ ,  $\alpha = 0.05$ ), 4 dpe ( $U = 0$ ,  $P = 0.0005$ ,  $\alpha = 0.05$ ), and 7 dpe ( $U = 0$ ,  $P = 0.0005$ ,  $\alpha = 0.05$ ) with no difference in sperm count at 14 dpe (figure 3.9).

Atrazine treatments of the wild larvae showed no significant difference in sperm count at any age point (figure 3.10).

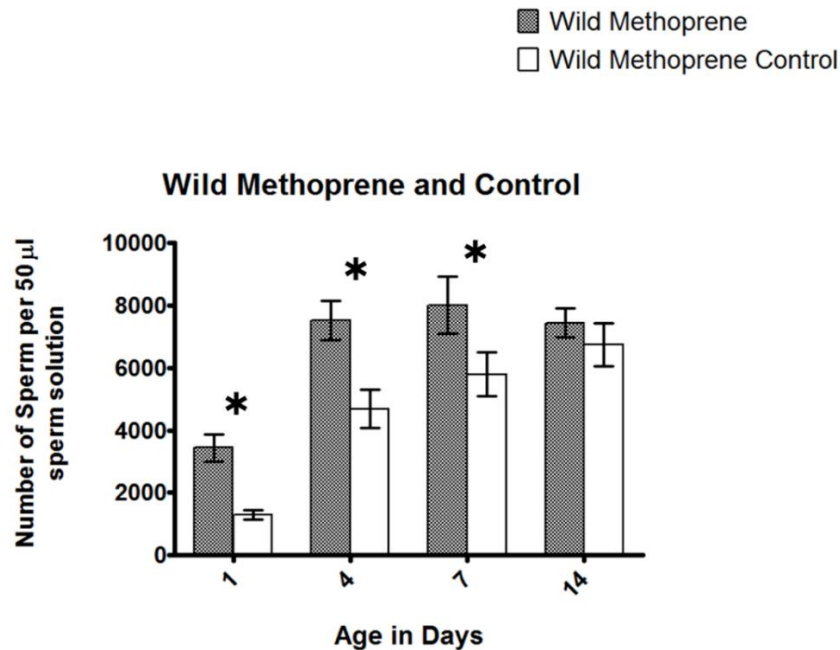
Glyphosate treatments of the wild larvae showed no significant difference in sperm count at any age point (figure 3.11).



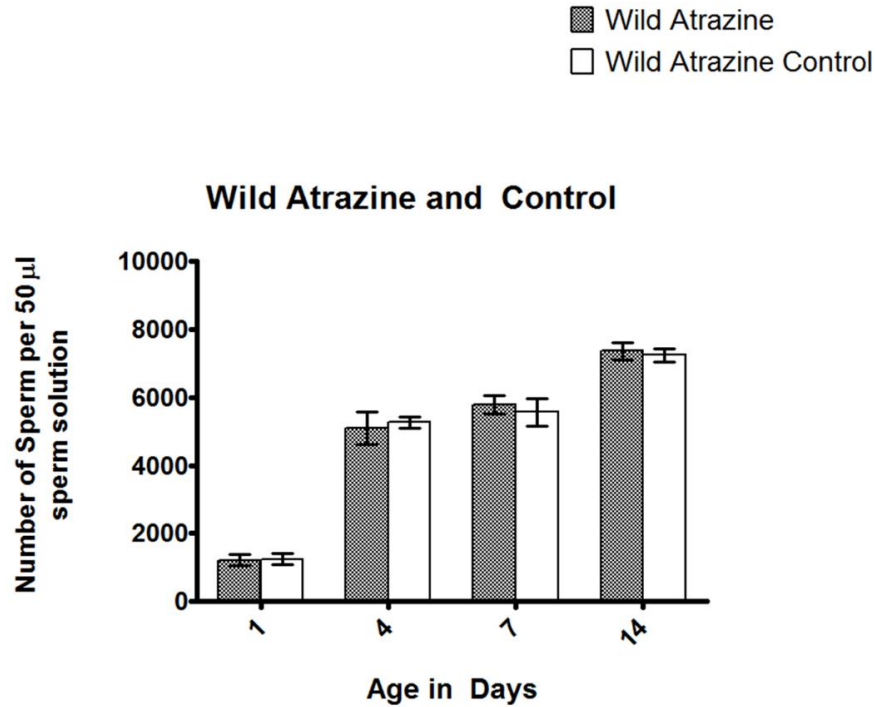
**Fig 3.7.** Average number of sperm per 50µl of sperm solution for wild *Culex quinquefasciatus* treated with spinosad by age in days ( $N = 60$ ) and control ( $N = 24$ ). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .



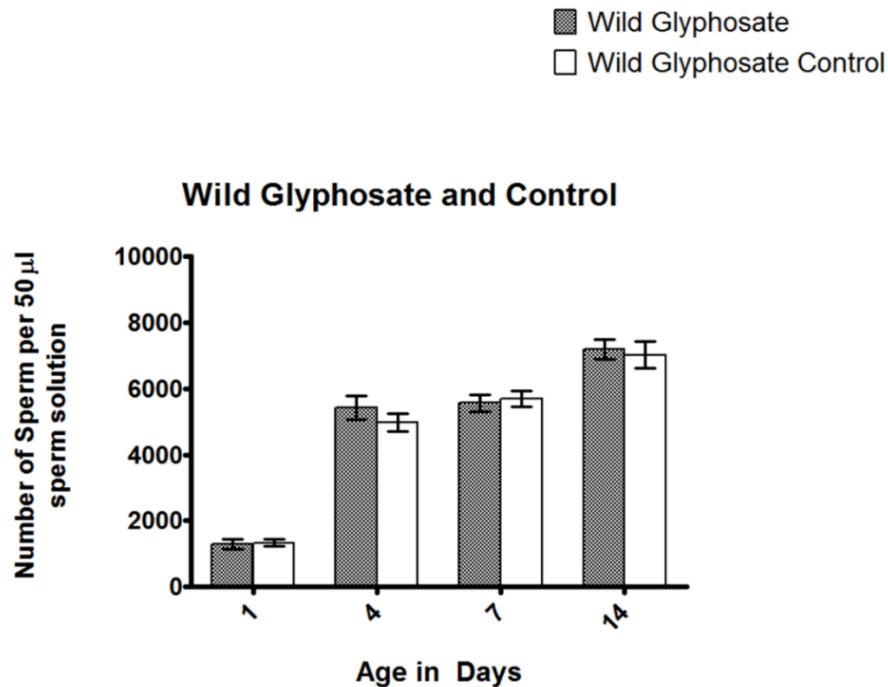
**Fig 3.8.** Average number of sperm per 50µl of sperm solution for wild *Culex quinquefasciatus* treated with *Lysinibacillus sphaericus* by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .



**Fig 3.9.** Average number of sperm per 50 µl of sperm solution for wild *Culex quinquefasciatus* treated with methoprene by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .

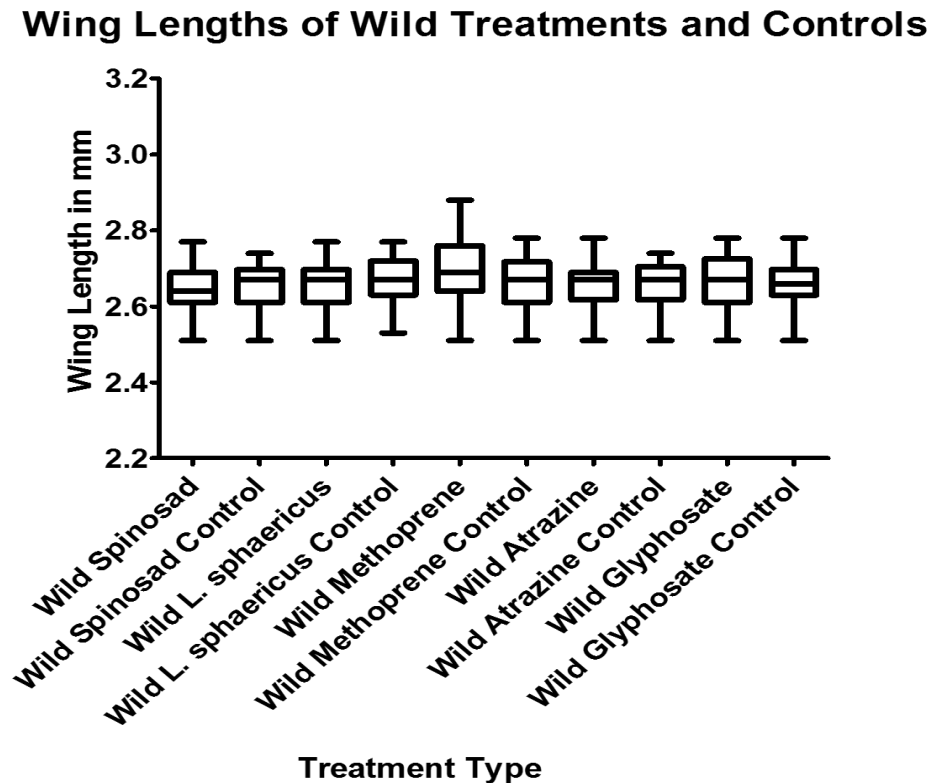


**Fig 3.10.** Average number of sperm per 50 µl of sperm solution for wild *Culex quinquefasciatus* treated with atrazine by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .



**Fig 3.11.** Average number of sperm per 50 µl of sperm solution for wild *Culex quinquefasciatus* treated with glyphosate by age in days (N = 60) and control (N = 24). Treatment and control bars of the same age marked with an asterisk are statistically significant from each other with  $\alpha = 0.05$ .

The nonparametric Kruskal-Wallis one-way ANOVA was used to determine differences in wing length among wild treatments and controls. There was no overall statistical significance, nor any specific differences after using the post hoc Dunn's test to compare each individual treatment to its control (figure 3.12).



**Fig 3.12.** Average wing length for wild *Culex quinquefasciatus* (N = 420). Plots with different letters are statistically significant with  $\alpha = 0.05$ .

### 3.4 Discussion

The first hypothesis of this study was that sperm production of wild collected and laboratory reared mosquitoes would be negatively affected by larval exposure to sub-lethal concentrations of spinosad, *Lysinibacillus sphaericus*, methoprene, atrazine, and glyphosate. As the results of each treatment varied from one to the next, it is difficult to say decisively that the null hypothesis (that exposure would not negatively impact sperm count) should be rejected.

Results also varied among the two populations. Because of this high variability, the results of each treatment will be discussed individually.

The results from experiments conducted on laboratory colony mosquitoes help us understand basic reactions to pesticides; results from experiments conducted on wild mosquitoes help us understand practical field reactions to pesticides. The Sebring colony of *Culex quinquefasciatus* was first established in 1988, 30 years ago (Stancil 2000). With three decades of laboratory care protecting it from pesticide exposure, it is considered a naïve, pesticide susceptible strain of mosquito. Therefore, the effects of pesticide exposure on Sebring mosquitoes may be considered genuine, fundamental physiological responses for *Cx. quinquefasciatus*. Any observed effects, or lack thereof, may be useful in understanding underlying physiological reactions to pesticide exposure, but are not necessarily applicable to wild populations. The wild collected *Cx. quinquefasciatus* were collected as eggs throughout the summer and fall of 2017. The population from which these mosquitoes were sampled has been exposed to pesticide applications from the East Baton Rouge Mosquito Abatement and Rodent Control district, homeowner pesticide applications, and other unknown chemical stressors. It is possible that decades of potential pesticide exposure have selected for resistance traits in these local mosquitoes. The effects of any experiments on these wild mosquitoes provide us with a more realistic understanding of current field conditions.

Spinosad caused significant hypospermatogenesis in Sebring mosquitoes at 1 and 4 dpe, but there were no observed effects at 7 and 14 dpe. There was no observed effect on wild mosquitoes. Spinosad, after being ingested or absorbed through the cuticle, binds at the nicotinic acetylcholine receptors (nAChRs) (Jiang and Mulla 2009). Lethal levels of spinosad induce convulsions and paralysis prior to death (Salgado 1998). Sub-lethal exposure to spinosad caused

sperm cell malformations in *Rhynchophorus ferrugineus*, but sperm were not quantified in this study (Abdelsalam et al. 2016). While there are not many comparable studies examining the effects of spinosad on insect sperm production, experiments on other organisms may shed some light on this study's results. Nicotine, which also binds at nAChRs, has been shown to reversibly reduce sperm counts in rats (Oyeyipo et al. 2011). Rats fed a diet containing nicotine had significantly lower sperm counts than controls, but rats relieved of the nicotine laced diet resumed normal sperm production after 30 days. A similar effect was observed in the Sebring mosquitoes; seven days after leaving a spinosad contaminated environment, sperm production resumed normal levels. Perhaps the most compelling evidence to validate the results of the present study was also found in rats. After consuming a diet with 0.4% spinosad, male rats had significantly lower sperm counts and arrested sperm cell development (Yano et al. 2002). It is unknown what prevented hypospermatogenesis in the wild mosquitoes of this study, but point mutations and exposure to a number of xenobiotic factors have been shown to induce insecticide resistance and tolerance (Scott 1999, Enayati et al. 2005, Berticat et al. 2008). The results of this study add to the body of evidence that sub-lethal exposure to spinosad during spermatogenesis may reduce sperm counts in naïve populations.

*Lysinibacillus sphaericus* exposure had no observed effect on the sperm production of wild mosquitoes. The only significant effect from *L. sphaericus* was on the Sebring mosquitoes at 1 dpe. Spores produced by *L. sphaericus* are activated in the mosquito larvae's alkaline midgut, releasing cytotoxic proteins which attack the midgut epithelial cells (Tangsongcharoen et al. 2017). Previous work on sub-lethal exposure to *L. sphaericus* found delayed pupation and decreased adult longevity in *Cx. quinquefasciatus*, but no effects on reproductive capacity were noted. There is no other recorded evidence of *L. sphaericus* exposure directly affecting reproductive health. It can be

supposed then that sub-lethal exposure to *L. sphaericus* causes only minor somatic damage, requiring energy to be spent on recovering from that exposure at the cost of reproductive vigor. However, it is worth pointing out that this is the only treatment to have a significant difference at 1 dpe, with no differences at other ages. The rate of spermatogenesis has the greatest variability between individuals during the pupal stage (Mescher and Rai 1966). It is possible that the observed difference in this treatment at 1 dpe is due to residual variability from the irregularity of pupal sperm production.

Methoprene caused significant hyperspermatogenesis in both Sebring and wild mosquitoes at 1, 4, and 7 dpe, with both populations returning to normal levels by 14 dpe. Methoprene is an insect hormone mimic which, in lethal doses, indirectly kills insects by interrupting metamorphosis (Wu et al. 2006). Mosquitoes, exposed to sub-lethal concentrations of methoprene as larvae, have experienced reduced longevity, increased longevity, reduced fecundity, and delayed development of external male genitalia (Robert and Olson 1989, O'Donnell and Klowden 1997, Ritchie et al. 1997). The effects of methoprene exposure on spermatogenesis in mosquitoes have not previously been studied, but methoprene has been used to accelerate sexual maturation in fleas and fruit flies (Dean and Meola 1997, Bachmann et al. 2017).

Before attempting to explain the hyperspermatogenic effect of methoprene on *Cx. quinquefasciatus*, there are some important points to be made on spermatogenesis. First, the number of testicular germ cells, which mature and produce sperm, are predetermined during embryonic development (Dumser 1980). Second, spermatocyte divisions are not synchronous, resulting in waves of sperm production (Ndiaye et al. 1996). Based on these two facts, there are two ways methoprene could have caused such a rapid and prolonged increase in sperm production. First, the sub-lethal levels of methoprene could have caused an accelerated division of



spermatocytes. Second, the methoprene exposure could have recruited inactive germ cells and prematurely initiated meiosis. Knowing which of these phenomena occurred would require and ultrastructural review of spermatogenesis during methoprene exposure. How methoprene could have initiated either of these events is also unknown. However, two potential explanations are presented. JH titers increase significantly in male mosquitoes 2-3 dpe (Nouzova et al. 2018). This increase in JH titers corresponds with the steepest increase in sperm production in untreated Sebring mosquitoes. Sperm counts increased 171% from 1 to 4 dpe, and only 63% from 4 to 14 dpe. If this is a true correlation, JH may indeed play a direct role in spermatogenesis, a role which can be augmented by sub-lethal applications of the JH mimic methoprene. The second potential explanation, remembering that JH regulates nutritional tradeoffs in mosquitoes and directly controls ovarian nutrient allocation (Zhu and Noriega 2016), is that JH might also regulate nutrient allocation to the testes and methoprene artificially enhances nutrient delivery.

Perhaps more important than the how and why described above, two implications of these results are presented. First, if the methoprene enhanced sperm production observed in the laboratory also occurs in field conditions, a review of methoprene applications for mosquito control may be in order. Males exposed to sub-lethal concentrations at the end of a service period may have increased sperm counts. This could potentially negate the lethal effects of the treatment by them being able to more rapidly repopulate an area. This highlights the importance of applying methoprene at designated label rates, keeping clear records of application dates and areas, and applying methoprene at appropriate intervals for maximum control efficacy. Second, if the methoprene enhanced males are indeed capable of inseminating more females than non-treated males, methoprene could be a useful addition to SIT rearing strategies. Methoprene is already under consideration as part of the SIT rearing procedure for fruit flies (Tephritidae) (Bachmann et

al. 2017). In the case of mosquitoes, methoprene treated males would be capable of inseminating more females than wild males with lower sperm counts. If these males were sterile, through irradiation or other methods, the increased sperm count could potentially increase their efficacy. It is important to note, however, that the credibility of methoprene dosed mosquitoes as an SIT strategy is dependent upon a significant amount of further research. The results described above only suggest that methoprene may provide a sexual advantage due to the increased sperm count.

No effects were observed in either the Sebring or wild mosquitoes after exposure to atrazine. Previous experiments exposing mosquito larvae to atrazine failed to observe any negative effects in regards to male reproduction. The predominate effect of atrazine exposure to mosquito larvae, in published literature, is a decrease in their sensitivity to pesticides. *Aedes aegypti* larvae exposed to atrazine were significantly less sensitive to the pesticides *Bacillus thuringiensis israelensis*, and temephos (Boyer et al. 2006, Jacquet et al. 2015). Atrazine has the potential to increase aquatic microorganism densities (Muturi et al. 2013) which could result in greater food supply and thereby larger mosquitoes with higher sperm counts, but this effect was not observed in the present study.

No effects were observed in either the Sebring or wild mosquitoes after exposure to glyphosate. While glyphosate has been shown to reduce fecundity in spiders and lacewings, it is not known if these decreases in fecundity were due to lowered sperm counts (Schneider et al. 2009, Benamú et al. 2010). Glyphosate has also been implicated as a xenobiotic capable of inducing the expression of monooxygenase P450 enzymes, heightening mosquito tolerance to pesticide exposure (Riaz et al. 2009). Glyphosate is also capable of causing a significant number and variety of mutations in mosquito larvae DNA, though none so far that indicate any change in reproductive processes (Bansal and Chaudhry 2010). There is no evidence thus far to implicate glyphosate as a

chemical capable of reducing sperm in insects, and this research does not contribute any evidence toward the hypothesis that it could.

The second hypothesis of this study was that these chemical exposures would not affect body size, measured by wing length, and that any observed reduction in sperm would be caused by mechanisms other than retarded somatic growth. None of the treatment groups from either population experienced any significant changes in wing length when compared to their controls. These results do not describe any causality for increases or decreases in sperm count, but they do rule out simple body size related changes in sperm production.

This was the first study to measure the effects of larval exposure to pesticides on sperm production in mosquitoes. The effects of pesticide exposure varied by population and treatment. The Sebring mosquitoes experienced decreases in sperm count after being exposed to spinosad and *L. sphaericus*, increases in sperm count after being exposed to methoprene. None of the pesticide exposures negatively affected sperm count in the wild mosquitoes, but an increase was observed after methoprene exposure. None of the pesticide exposures had any significance on body size, as measured by wing length. These results provide us with a greater understanding of how pesticides may alter populations of mosquitoes by influencing the reproductive capacity of male mosquitoes. This serves as a reminder to the various pest control industries to be judicious in the use and frequency of pesticides. These results also provide background information which may be valuable in increasing the maximum reproductive output of male mosquitoes used in SIT releases, pending further research.

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## Summary and Conclusion

Mosquitoes are indisputable public health pests. The pathogens they vector are capable of causing disease, disfigurement, and death. The control of mosquito borne diseases can be accomplished through a variety of means: pesticide applications to control mosquito populations, vaccinations, habitat alterations, personal application of mosquito repellents, and more. The success of all of these strategies depends, in part, upon a thorough understanding of the biology of the mosquito vectors themselves. The more we know about the biology of a vector species, the better able we are to exploit their natural weaknesses and better protect public health.

Female mosquitoes, the only sex that blood feeds on and transmits pathogens to humans, are the primary targets of most control strategies. However, some strategies target mosquito mating as an opportunity to suppress mosquito populations. These strategies require a thorough understanding of the reproductive biology of both males and females. The female reproductive biology has been well studied, but many fundamental questions about male reproductive biology have gone unanswered. The goal of this study was to answer the following fundamental questions about male mosquito sperm production for the species *Culex quinquefasciatus*. Does sperm production increase with age? Do sperm production rates vary between laboratory mosquitoes and wild mosquitoes? Can sub-lethal pesticide exposure during the larval stage affect sperm production as an adult? The answers to these questions not only broaden general entomological knowledge of reproductive processes, they may also influence mosquito control practices. For example, the efficacy of any method of modified male release depends, in part, upon sexually competitive lab reared males.

The results of these studies showed that sperm production in *Culex quinquefasciatus* does continue with age, until at least 14 days post eclosion (dpe). The Sebring colony was shown to



produce more sperm than locally collected wild mosquitoes, especially at 14 dpe. Virgin males have significantly greater sperm capacity from 4-14 dpe than they do at 1 dpe. Pesticide exposure during the larval stage can impact sperm production as was seen in the spinosad, *Lysinibacillus sphaericus*, and methoprene treatments for the Sebring colony, and methoprene treatments for the wild collected mosquitoes. Both spinosad and methoprene have been implicated in other experiments as having the potential to interrupt or enhance sperm production and other reproductive processes. The cumulative results of this study add to the currently small body of knowledge concerning mosquito sperm production and some external factors which may influence that production.

The most compelling result of this study was the significant increase in sperm production in both the Sebring and wild mosquitoes after exposure to methoprene. There are two significant implications to be made from this discovery. First, if applications of methoprene become inconsistent, or are not well tracked, mosquito populations may actually benefit, over time, from sub-lethal exposure due to their potentially increased reproductive ability. Second, if the methoprene enhanced sperm counts occur in conjunction with a male's ability to successfully inseminate more females than an untreated male, further researcher could use these findings to manipulate mosquito reproductive biology to better control mosquitoes.

As this research focused on answering fundamental questions about sperm production in *Culex quinquefasciatus* mosquitoes, many crucial questions remain unanswered. We learned more about sperm production at different ages and that sub-lethal levels of some pesticides interact with that process, but other important factors should be evaluated. Would these chemical exposure have similar effects on other mosquito species? There are other larvicides used against mosquitoes that could be evaluated. Would LC<sub>20</sub> treatments amplify or diminish the effects observed in this

research? Do any of these treatments, whether or not they affected sperm production, affect the viability of the sperm produced? How would results from semi-field studies differ from the current results, and what applications for control would they have? The ground work has been laid so that these questions, and others, can be answered.

## **Vita**

Shiloh Judd was born in 1989 in Tucson, Arizona and raised in Garner, North Carolina. After graduating from high school in 2007 he spent two years in Malaysia and Singapore as a missionary for the Church of Jesus Christ of Latter-day Saints. Beginning in 2011 he attended Brigham Young University-Idaho and earned his bachelor's degree in Health Science with an emphasis in Public Health, graduating in 2015. In that same year he began his Master of Science degree in Entomology at Louisiana State University in Baton Rouge, Louisiana advised by Dr. Kristen Healy. During this time he served as the Co-chair and Chair of the Young Professionals Committee within the American Mosquito Control Association. He is planning to graduate in 2018 and is planning to accept a commission as a 1<sup>st</sup> Lieutenant in the United States Army as a Medical Entomologist.