ABSTRACT

DE JESUS, CARRIE ELIZABETH. What is the Purpose of Multiple Lobed Spermathecae in *Aedes aegypti* and *Aedes albopictus*? (Under the direction Dr. Michael Reiskind).

Aedes aegypti and *Aedes albopictus* are two medically important mosquito species. *Aedes aegypti* and *Ae. albopictus* are both competent vectors of dengue and chikungunya and threaten 2.5 billion in the tropics and subtropics. *Aedes aegypti* and *Ae. albopictus* share similar morphological, ecological, and behavioral characteristics. Few studies have examined differences in their reproductive physiology. One trait that both species share is the presence of a multiple lobed spermathecae. Both species have one medial lobe and a paired set of lateral lobes. Evidence in other dipteran species have found that sperm can asymmetrically distribute between multiple lobes and sperm from particular lobes can be selected for fertilization. *Aedes aegypti* and *Ae. albopictus* sort their sperm asymmetrically between their three lobes. However, the importance of multiple lobed spermathecae has yet to be investigated in mosquitoes. In this thesis I examined possible usages of the three lobed spermathecae in *Ae. aegypti* and *Ae. albopictus*. I investigated if male body size influenced spermathecal filling, if heterospecific mating influenced lobe filling and if females could select which lobes participate in fertilization. I also investigated aspects of precopulatory behaviors in conspecific and heterospecific matings in *Ae. aegypti* and *Ae. albopictus*. I found that male body size did not influence the number of spermathecal lobes filled in either species. Heterospecific mating did not influence which lobes sperm was stored in. For sperm depletion no evidence was found that females of either species had a preference for the use of either the medial or lateral lobe. Sperm depletion over multiple gonotrophic cycles was seen in *Ae. aegypti* but not for *Ae. albopictus*. The largest difference in sperm depletion was seen over multiple gonotrophic cycles in *Ae. aegypti*. The medial lobe had the greatest decrease in

sperm after 2 cycles, while the lateral lobe had some depletion detected. However *Aedes albopictus* stored more sperm than *Ae. aegypti* overall and laid more eggs. For both species, females laid more eggs when they mated with large males versus small males. I also observed precopulatory behaviors in conspecific and heterospecific mating with both species. *Aedes aegypti* males attempted to mate with females more often than *Ae. albopictus* in conspecific matings regardless of body size. In heterospecific matings fewer matings occurred overall. Both *Ae. aegypti* and *Ae. albopictus* do sort their sperm asymmetrically between spermathecal lobes. However body size, heterospecific mating and sperm lobe selection did not play a role in spermathecal filling or depletion. Future studies should be conducted to examine precopulatory and postcopulatory activities that may influence spermathecal filling and reproductive mating behaviors.

What is the Purpose of Multiple Lobed Spermathecae in *Aedes aegypti* and *Aedes albopictus?*

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Masters of Science

Entomology

Raleigh, North Carolina

2015

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DEDICATION

To my loving grandparents Ralph and Shirley Klingbeil

BIOGRAPHY

Carrie De Jesus moved across the country from Southern California to NCSU to be part of the Entomology Graduate program. Carrie received her Bachelor's degree in Biology at California State University Fullerton. After she obtained her Bachelor's she was hired as a technician on West Nile Virus project in Central California. During her time as a technician she did surveillance work collecting thousands of mosquito samples and obtained many mosquito bites as well. This experience sparked her interest in entomology. Shortly after she worked as a vector control technician. Carrie was directly involved treating mosquito infested areas and interacting with the public to inform them about disease vectors. She still wanted to continue conducting research and wanted to but focus in vector biology.

NCSU's strong entomology program and Dr. Reiskinds interest in mosquito behavior combined made NCSU the perfect choice to obtain her Master's degree. Carrie joined the Reiskind lab in 2013 and is researched mating behavior and reproductive physiology in *Aedes aegypti* and *Aedes albopictus*.

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Michael Reiskind for his support and guidance throughout the course of my graduate degree. I also like to thank all of the Reiskind lab members for their assistance in various portions of my project. A special thanks to Hannah Jenkins who has been a wonderful undergraduate research assistant. I am also thankful for the support of family even though I was 2,541 miles away.

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Introduction:

Female mosquitoes are responsible for the transmission for a wide array of pathogens that can impact human health. Well known pathogens transmitted by mosquitoes include malaria, lymphatic filarial worms, dengue, and chikungunya. Dengue and chikungunya are two emerging viral diseases that threaten approximately 2.5 billion people in the tropics and subtropics (Halstead 2007, Gratz 2004). Currently there is no vaccine or clinical treatment available for these infections (Webster et al. 2009).

Aedes aegypti and *Aedes albopictus* are the two most important disease vectors for dengue and chikungunya viruses (Gubler 2002). *Aedes aegypti* and *Ae. albopictus* are invasive species that have spread to all continents except for Antarctica (Juliano and Lounibos 2005). These species did not initially overlap in their geographic distribution. *Aedes aegypti* originates from Africa while *Ae. albopictus* is native to Asia. *Aedes aegypti* was introduced to the Americas in the late 1700's on trade ships (Christophers 1960) where it has since been naturalized. *Aedes albopictus* was introduced to United States in the 1980's from tires imported from Asia, where it then rapidly spread across the South Eastern United States and displaced populations of *Ae. aegypti* (Hobbs et al. 1991; O'Meara et al. 199).

Aedes aegypti and *Ae. albopictus* are both in the subgenus *Stegomyia* and are morphologically similar to each other. Both species are small black and white mosquitoes, with bands of white scales throughout their body and legs. *Aedes aegypti* has a lyre shaped white scale pattern on its scutum, while *Ae. albopictus* has a white scale stripe on its scutum. *Aedes aegypti* was white scaled palps while *Ae. albopictus* has dark palps (Burkett-Cadena, 2013).

Aedes aegypti and *Ae. albopictus* share a variety of behavioral characteristics. Both species are diurnally active. They both oviposit in natural containers (tree holes) or artificial containers (flower pots, bird baths, tires, etc.). Both species aggregate and mate near their hosts, often humans or other mammals (Clements 1999). Because *Ae. aegypti* and *Ae. albopictus* are similar to one another in many aspects and have demonstrated a pattern of competitive displacement, comparative studies are needed to delineate the ecological differences that may be responsible for competitive displacement. One set of differences may be in their larval competitiveness and ability to resist desiccation (Juliano 2010, Costanzo et al. 2005, Juliano et al. 2002). A second possibility are differences in their mating biology, which is the focus of this thesis.

The similarities and differences in mating biology between *Ae. aegypti* and *Ae. albopictus* have not been thoroughly examined. The majority of studies of *Aedes* mosquito mating biology date back to 1970's or earlier (Oliva et al. 2014). A resurgence in interest in mating biology has occurred in this past decade due to potential use of genetic control techniques (Helinski et al. 2012a, Oliva et al. 2014). To use these genetic techniques in the future, a thorough understanding of mating biology is required. Further investigations in mating biology are needed to develop new tools for vector control.

General descriptions of how matings occur have been documented in the literature (Clements 1999). Matings occur near a host where males and females congregate for both species. In both *Ae. aegypti* and *Ae. albopictus*, males fly or hover around or near the host till they find a potential mate. Mating is initiated in flight, followed by visual and auditory signals (Roth, 1948, Cator et al. 2009). Males will attempt to grasp onto females during flight

(Roth 1948). Females that are not receptive to mating will fly away from males (Roth 1948). Females that are responsive to males will allow males to position themselves venter to venter (Roth 1948). Copulation in *Ae. aegypti* and *Ae. albopictus* can take place on the host or in flight (Roth 1948, Gubler and Bhattacharya 1972, Hartberg 1971). In the venter to venter position males flex there abdomen upward to make contact with the female genitalia in both *Ae. aegypti* and *Ae. albopictus* (Roth 1948, Oliva 2014). The male's genitalia then interlocks with female genitalia. *Aedes aegypti* pairs can remain interlocked for 9 to 31 seconds, while in *Ae. albopictus* copulation can last 30-50 seconds (Roth 1948, Jones and Wheeler 1965a; Olivia et al., 2014). Once interlocked males will transfer sperm to the female. When copulation is completed females will separate from the male and fly away in *Ae. aegypti* and *Ae. albopictus* (Roth, 1948; Gubler and Bhattacharya, 1972).

Recent investigations have found that other mating behaviors may be at play before and after copulation. Precopulatory behaviors that have been studied in *Aedes* have focused on an aggregation pheromone and harmonic convergence. In *Ae. aegypti* mating pairs modulate their flight tones to harmonize with each other before copulation (Cator et al. 2009, Cator and Harrington, 2011). Male (557- 750Hz) and female (330–544Hz) *Ae. aegypti* have different fundamental flight tones. Both sexes have the ability to modulate their flight tones. During flight a mating pair can match their flight tone to converge at harmonic frequencies (Cator et al. 2009, Cator and Harrington 2011). Mating pairs that participated in harmonic convergence were more likely to copulate than pairs that did not (Cator and Harrington 2011). The male offspring of pairs that participated in harmonic convergence had increased mating success and also participated in harmonic convergence (Cator and Harrington, 2011).

Harmonic convergence has been document in other Culicidae species: *Anopheles gambiae* and *Culex pipens* (Pennetier et al. 2010, Warren et al. 2009). Another precopulatory behavior that has been examined in *Ae. aegypti* is the presence of an aggregation pheromone (Fawaz et al. 2014; Cabrera and Jaffe, 2007). *Aedes aegypti* males produce a volatile pheromone that induces flight activity in females leading to swarming behavior (Cabrera and Jaffe, 2007). Precopulatory behaviors in *Ae. albopictus* have not been well investigated.

Post-copulatory mating behaviors have become a focus in mosquito mating biology as well, although the emphasis has been placed on male ejaculate. Males produce a variety of seminal protein fluids (Spfs) that are transferred to the female during copulation. Seminal protein fluids are responsible for behavioral and physiological changes in female insects (Avila et al. 2011). Examples of changes due to Spfs include: refractoriness to mating, increased egg production, sperm viability and, sperm storage. Seminal protein fluids for *Ae. aegypti* and *Ae. albopictus* cause refractoriness to further mating up to 34 days after mating (Helinski et al. 2012a). Females after insemination become unreceptive to mating attempts for at least 1 gonotrophic cycle in *Ae. aegypti* (Young and Downe 1982). Refractoriness has been documented in other mosquito species such as *Anopheles gambiae*. Accessory gland proteins in *An. gambiae* and other anopheiline species are responsible for the formation of a mating plug (Mitchell et al. 2015). In *An. gambiae* the Spfs are required for sperm storage (Rogers et al. 2009). Rogers et al. (2009) studied the composition of the anopheline mating plug. They found that mating plug is formed the cross-linkage of seminal protein fluids with a MAG-specific transglutaminase (TGase) (Rogers et al. 2009). In *An. gamibae* females that mated with TGase inhibited males did form a mating a plug which prevented sperm storage.

Seminal protein fluids may also be vital to sperm viability in the spermathecae, based upon their role in other insect taxa. Seminal fluid proteins in the honeybee, *Apis mellifera*, the leafcutter ant *Atta colombica*, and *Drosophila melanogaster* promoted sperm viability in sperm storage organs (Avila et al. 2011). Also in *D. melanogaster*, accessory gland proteins (Acps) are needed to store and utilize sperm. One investigation knocked out 5 different Acps in *D. melanogaster* which resulted in females retaining sperm instead of utilizing it for fertilization. Egg production has also been linked to increased egg production in a variety of insect taxa. In the Lepidopteran species *Helicoverpa armigera* male accessory gland extracts stimulated egg maturation and oviposition (Jin and Gong 2001, Avila et al. 2011). In *D. melanogaster* 4 Spfs have been identified that stimulate egg laying in mated females (Gillott, 2003, Ram and Wolfner 2007). Seminal protein fluids have been linked to fecundity in *An. gambiae* (Ekbote et al. 2003, Isaac et al. 2007, Avila et al., 2011). Male *An. gambiae* have an angiotensin converting enzyme in their reproductive glands (Issac et al., 2007). When the production of this enzyme is inhibited, females that mated with inhibited males laid fewer eggs than those that did not (Issac et al. 2007). In *Ae. aegypti* the Spfs increase oviposition, but it is unknown if this occurs in *Ae. albopictus* as well (Gillott, 2003, Sirot et al. 2008). While Spfs play an important role in postcopulation behavior of female insects, they do not influence the entrance and exit of sperm from the spermathecae (Avila et al., 2011). Female mosquitoes still have the ability to influence sperm intake and sperm usage in egg fertilization.

One part of the female reproductive tract that has received little attention in the past few decades is the spermatheca, the sperm storage organ in female insects. The spermatheca

is used for long term-storage, can allow for multiple bouts of oviposition in a wide range of insect species and can have a variety of structures (Twig and Yuval, 2005, Taylor and Yuval, 1999). Within Culicidae, species vary in the number of spermathecal lobes present. *Anopheles* have 1 lobe, some *Culex* have 2 lobes, while *Aedes* can have between 1 to 3 lobes (Clements 1999, Reidenbach et al. 2009). *Aedes aegypti* and *Ae. albopictus* both have a three lobed spermathecae.

The structure of the spermathecae has been well characterized for *Ae. aegypti* but not for *Ae. albopictus* (Clements and Potter 1967, Pascini et al. 2012) However they are superficially similar to one another. Both species have one medial lobe (diameter: 100um) and two smaller lateral lobes (diameter: 75um). All three lobes are attached to the common spermathecal duct. However the medial lobe splits off into its own duct away from the lateral lobes, off the common duct. The lateral lobes share a duct that then splits into two smaller ducts leading to each lobe (Fig.1.1).

Sperm are transported to the lobes through the ducts through a fluid withdraw mechanism (Linely and Simmons 1981). In *Ae. aegypti* it is hypothesized that the periductal glandular cell structure is similar to cells that participate in liquid transportation and that the capsular glandular cells have organelles that may function in liquid transportation (Clements 1999). Dye injections demonstrated that fluids from the bursa were moved to the spermathecal lobes in females inseminated when alive (Wheeler and Jones, 1965b). In dead mated females dyes were not transported to the spermathecal lobes. This indicates that sperm motility alone cannot reach the lobes but another mechanism is required to move sperm to the spermathecae by the female (Linely and Simmons 1981).

Linely and Simmons (1981) provide multiple examples of why sperm motility alone is not a plausible mechanism of the sperm transportation to the spermathecal lobes. First, sperm do not swim fast enough on their own to account for the rate of which sperm fill the spermathecal lobes. Second, sperm entering the capsule would displace fluid creating a counter current. This current would therefore increase the amount of speed needed to reach the lobes. Finally, the propelling motion of the sperm tail is too large to pass through the spermathecal ducts.

Sperm are transported up the ducts until they are stored in one of the three spermathecal lobes. Within the lobes sperm are packed in parallel bundles to maximize sperm storage (Pascini et al. 2012). Sperm of *Ae. aegypti* are long (200-250um) and thin (diameter 0.5-0.6um) (Fig.1.2) (Klowden and Chambers 2004). The bundles of sperm within the lobe move in a circular motion to prevent clumping and allow sperm to exit the spermathecae one by one (Lefevre and Jonsson 1962, Al-Lawati et al. 2009). The main purpose of the spermathecae is the long-term sperm storage until the female can oviposit. Sperm can be stored in the spermathecae for days to months (Clements 1999). Glandular cells are present around each lobe which have been shown to produce sugars that may play a role in preserving sperm (Pascini et al. 2012). These sugars are produced in both inseminated and not inseminated females (Pascini et al. 2012). The medial lobe has double the number of glandular cells compared to the lateral lobes (Pascini et al. 2012). Jones and Wheeler observed the number of sperm stored in the lobes of the spermathecae and suggest that approximately 660 sperm can fit in the medial lobe and 486 in a lateral lobe (Wheeler and Jones, 1965a).

When females oviposit, the egg is fertilized right before it is deposited (Clements 1999). *Aedes aegypti* laterally contract their eighth abdominal segment many times that causes movement of the spermathecae (Clements 1999). Each lobe has a sphincter at the base that opens and closes to allow the entrance and exit of sperm. Each sphincter can be opened and closed independently from other lobes (Pascini et al. 2012). One example of muscular control of the transportation of sperm is seen in the bee *Megachile rotundata* in which females stop abdominal contraction for oviposition of male, haploid offspring (Gerber and Klostermeyer 1970). A pause in contractions is made which allows the use of the sphincter to transport sperm to the egg to produce female offspring (diploid). This mechanism therefore allows the female to have control over the fertilization or non-fertilization of her offspring (Gerber and Klostermeyer 1970). This may allow females with multiple lobes to select which lobe to fertilize eggs with. These characteristics of the spermatheca indicate that female mosquitoes have control over spermathecal filling and egg fertilization. How the three lobes of the spermathecae play a role in the mating biology of *Ae. aegypti* and *Ae. albopictus* has not been examined.

Multiple sperm storage organs are common in a variety of arthropod taxa including millipedes, crustaceans, spiders and ticks. (Eberhard 1996). Multiple spermathecae are commonly seen throughout Diptera and typically range from 2-4 lobes (Eberhard 1996). Female insects have physiological and neuromuscular controls that allow females to determine where sperm will be stored without influence by the male (Eberhard 1996). The use of multiple sperm storage organs has been examined in various dipteran species. Lobe filling is dependent on muscular control of the female reproductive tract in dipterans (Linley

and Simmons 1981). Female *Drosophila melanogaster* actively transport sperm from the ventral receptacle to the spermathecae during and after copulation (Arthur et al. 1998). Arthur (1998) reared females with damaged or undamaged nervous systems. The females with an altered nervous system moved significantly less sperm to the spermathecae when compared to unaltered females. Arthur's result implies that females play a crucial role in the transportation of sperm to the spermathecae, and, in the case of multiple spermathecae, have control over which lobes to fill.

Polyandrous and monadrous dipteran species have the displayed the ability to distribute sperm unevenly amongst multiple spermathecal lobe. *Scathophaga stercoraria*, the yellow dung fly, are polyandrous and store sperm from two to three males (Ward 1993, Ward 2000, Demont et al. 2012). Evidence of female cryptic choice has been well investigated in *S. stercoraria*. Female cryptic choice is the ability for a female to select a male's sperm after insemination (Eberhard 1996). In a study by Ward (1993) females were mated with both large and small sized males in sequence. Sperm from larger males that mated first were stored in the paired lobes, which allowed consecutive egg batches from the larger male. Another study found that females who mated twice, the sperm from the second male was stored in the singlet spermathecae and not in the doublet (Bussiere, et al., 2010; Ward, 1993). When females mated with larger males second the larger male did not deposit as much sperm compared to the smaller male. However females still selected the sperm of large male over the small but produced less offspring (Ward, 1993). Ward's study provides evidence for female cryptic choice, by demonstrating the female's ability to select sperm for egg fertilization after copulation. *Anastrepha suspense* (the Caribbean fruit fly) is also a

polyandrous species that has a 3 lobed spermathecae with a paired set and a singlet. Studies that investigated *A. suspense*, found that sperm were distributed asymmetrically amongst all three lobes (Fritz 2004, 2009). Sperm were typically stored in the paired lobes and rarely filled the singlet lobe (Fritz, 2009). *Ceratitis capitata* (the Mediterranean fruit fly) also asymmetrically distributed sperm amongst two spermathecal lobes (Taylor and Yuval 1999, Twig and Yuval 2005). *Bactrocera tryoni* (the Queensland fruit fly) is monandrous and has 2 spermathecae. Females mate with males once and become refractory to mating similar, to *Ae. aegypti* and *Ae. albopictus*. Female *B. tryoni* after mating are able to oviposit up to 7 weeks after mating indicating they can store and utilize sperm over long spans of time (Perez et al. 2007). Asymmetrical spermathecal filling is present in *B. tryoni* relative the amount of sperm deposited. In spite of the several examples across Diptera, the reasons why sperm are asymmetrically distributed is unclear.

As stated previously *Ae. aegypti* and *Ae. albopictus* often have asymmetrical lobe filling by only using the medial and one lateral lobe. Both *Ae. aegypti* and *Ae. albopictus* are typically considered monandrous species, but recent studies have shown that polyandry does occur in both species at low rates (10-14%) (Helinski et al. 2012b, Boyer et al., 2012). How mosquitoes utilize multiple sperm storage organs has yet to be investigated.

As *Ae. aegypti* and *Ae. albopictus* are two medical important mosquitoes, further investigation into the roles of the spermathecal lobes could provide new insight on their mating and reproductive biology. For this investigation multiple aspects of spermathecal lobe filling and mating behavior were examined: lobe filling as related to male body size and heterospecific matings, premating behavior, and the depletion of sperm over time. The

following chapters will present the results of a series of experiments examining these topics in *Ae. aegypti* and *Ae. albopictus*.

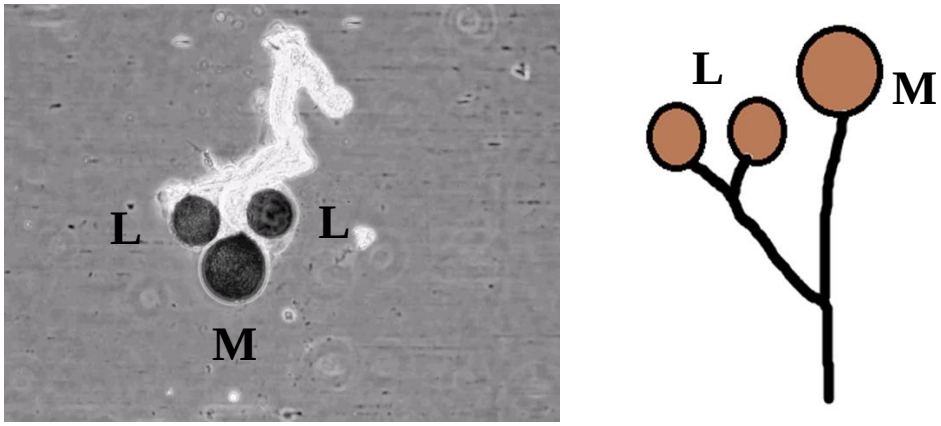

Figure 1.1 A) The three lobed spermathecae of *Aedes albopictus* at 200X using phase contrast. B) Depication of spermathecal lobes showing the splitting of the spermathecal ducts to the medial and lateral lobes (not to scale). M = Medial Lobes, L = Lateral Lobe.

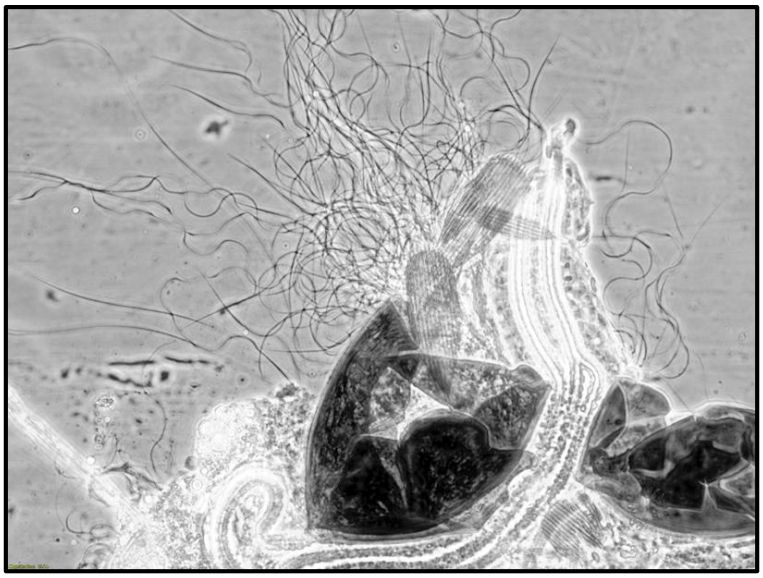

Figure 1.2 Sperm swimming out of a crushed spermathecal lobe. Phase Contrast 200x

References

Al-Lawati, H., Kamp, G., and Bienefeld, K. 2009. Characteristics of the spermathecal contents of old and young honeybee queens. Journal of insect physiology, 55(2), 117-122.

Arthur, B., Hauschteck-Jungen, E., Nöthiger, R., and Ward, PI. 1998. A female nervous system is necessary for normal sperm storage in *Drosophila melanogaster*: a masculinized nervous system is as good as none. Proceedings of the Royal Society of London B: Biological Sciences, 265(1407), 1749-1753.

Avila, F., Sirot L., LaFlamme B., Rubinstein C., Wolfner M. 2011. Insect seminal fluid proteins: Identification and function. Palo Alto: Annual Reviews. 56(1): 21-40.

Boyer, S., Toty, C., Jacquet, M., Lempérière, G., Fontenille, D. 2012. Evidence of multiple inseminations in the field in *Aedes albopictus*. PloS One 7(8):e42040.

Burkett-Cadena, N. D. 2013. Mosquitoes of the southeastern United States. University of Alabama Press.

Bussiere, L., Demont M., Pemberton A., Hall, M., Ward P. 2010. The assessment of insemination success in yellow dung flies using competitive PCR. Molecular Ecology Resources 10(2):292-303.

Cabrera, M., Jaffe, K., 2007. An aggregation pheromone modulates lekking behavior in the vector mosquito *Aedes aegypti* (Diptera: Culicidae). J. Am. Mosq. ControlAssoc. 23, 1–10.

Cator, L.J., and Harrington, L.C. 2011. The harmonic convergence of fathers predicts the mating success of sons in *Aedes aegypti*. Anim Behav 82(4):627-633.

Cator, L.J., Arthur, B.J., Harrington, L.C., Hoy, R.R. 2009. Harmonic convergence in the love songs of the dengue vector mosquito. Science 323(5917):1077-1079.

Christophers, S.R. 1960. *Aedes aegypti* (L.), the yellow fever mosquito: Its life history, bionomics, and structure. Cambridge ; New York, N.Y.: Cambridge University Press.

Clements, A.N., and Potter S.A. 1967. The fine structure of the spermathecae and their ducts in the mosquito *Aedes aegypti*. J Insect Physiol 13(12):1825-36.

Clements, A.N. 1999. The biology of mosquitoes. Wallingford, Oxon, UK ; Cambridge, MA: CABI.

Costanzo, K.S., Kesavaraju, B., and Juliano, S.A. 2005. Condition-specific competition in container mosquitoes: the role of noncompeting life-history stages. Ecology, 86(12), 3289-3295.

Demont, M., Buser, C.C., Martin, O.Y, Bussière, L.F. 2011. Natural levels of polyandry: Differential sperm storage and temporal changes in sperm competition intensity in wild yellow dung flies. Funct Ecol 25(5):1079-90.

Eberhard, W.G. 1996. Female control: Sexual selection by cryptic female choice. Princeton, N.J: Princeton University Press.

Ekbote, U., Looker, M., Isaac, R.E. 2003. ACE inhibitors reduce fecundity in the mosquito, *Anopheles stephensi*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 134:593–98.

Fawaz,E.Y,. Allan, E.A., Bernier, U.R., Obenauer, P.J., and Diclaro, W.J II. (2014). Swarming mechanisms in the yellow fever mosquito: Aggregation pheromones are involved in the mating behavior of *Aedes aegypti*. Journal of Vector Ecology, 39(2), 347-354.

Fritz, A. 2009. Sperm storage is not subject to cephalic control in the caribbean fruit fly, *Anastrepha suspensa*. J Insect Behav 22(5):412-22.

Fritz, A.H. 2004. Sperm storage patterns in singly mated females of the caribbean fruit fly, *Anastrepha suspense* (Diptera: Tephritidae). Ann Entomol Soc Am 97(6):1328-35.

Gillott, C. 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annu. Rev. Entomol. 48:163–84.

Gratz, N.G. 2004. Critical review of the vector status of *Aedes albopictus*. Medical and Veterinary Entomology, 18(3), 215-227

Gerber, H.S., and Klostermeyer E.C. 1970. Sex control by bees: A voluntary act of egg fertilization during oviposition. Science (New York, N.Y.) 167(3914):82-4.

Gubler, D.J., and Bhattacharya N.C. 1972. Swarming and mating of *Aedes*-(*S*)-*albopictus* in nature. Mosquito News 32(2):219-223.

Gubler, D.J. 2002. Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. Trends Microbiol 10(2):100-103.

Isaac, R.E., Lamango, N.S., Ekbote, U., Taylor, C.A., Hurst, D., et al. 2007. Angiotensin-converting enzyme as a target for the development of novel insect growth regulators. Peptides 28:153–62.

Halstead, S.B. 2007. Dengue. The Lancet, 370(9599), 1644-1652.

Hartberg, W.K. 1971. Observations on the mating behaviour of *Aedes aegypti* in nature. Bull World Health Organ 45(6):847-850.

Helinski, M.E., Deewatthanawong, P., Sirot, L.K., Wolfner, M.F., Harrington, L.C. 2012a. Duration and dose-dependency of female sexual receptivity responses to seminal fluid proteins in *Aedes albopictus* and *Ae. aegypti* mosquitoes. J Insect Physiol 58(10):1307-1313.

Helinski, M.E., Valerio, L., Facchinelli, L., Scott, T.W., Ramsey, J., Harrington L.C. 2012b. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. Am J Trop Med Hyg 86(4):635-641.

Hobbs, J., Hughes, E., Eichold B. 1991. Replacement of *Aedes-aegypti* by *Aedes-albopictus* in Mobile, Alabama. J Am Mosq Control Assoc 7(3):488-489.

Jin, Z.Y and Gong, H. 2001. Male accessory gland derived factors can stimulate oogenesis and enhance oviposition in *Helicoverpa armigera* (Lepidoptera: Noctuidae). Arch. Insect Biochem. 46:175–85.

Jones, J.C., and Wheeler R.E. 1965a. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. description. Biol Bull 129(1):134-150.

Jones, J.C., and Wheeler, R.E. 1965b. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). II. experimental. Biol Bull 129(3):532-545.

Juliano, S.A. 2010. Coexistence, exclusion, or neutrality? A meta-analysis of competition between Aedes albopictus and resident mosquitoes. Israel journal of ecology & evolution, 56(3-4), 325-351.

Juliano, S.A., and Lounibos P.L. 2005. Ecology of invasive mosquitoes: Effects on resident species and on human health. Ecol Lett 8(5):558-574.

Juliano, S.A., O'Meara, G.F., Morrill, J., Cutwa, M., Braks, MAH., and Honorio, N.A. 2002. Competition and co-existence of *Aedes albopictus* and *Aedes aegypti*.Klowden, M. J., and Chambers, G. M. (2004). Production of polymorphic sperm by *Anopheline* mosquitoes and their fate within the female genital tract. Journal of Insect Physiology, 50(12), 1163-1170.

Lefevre Jr, G., and Jonsson, U.B. 1962. Sperm transfer, storage, displacement, and utilization in *Drosophila melanogaster*. Genetics, 47(12), 1719.

Linley, J., and Simmons, K. 1981. Sperm motility and spermathecal filling in lower Diptera. International Journal of Invertebrate Reproduction 4(2):137-145.

Mitchell, S.N., Kakani E.G., South, A., Howell, P.I., Waterhouse, R.M., Catteruccia, F. 2015. Mosquito biology. Evolution of sexual traits influencing vectorial capacity in *Anopheline* mosquitoes. Science (New York, N.Y.) 347(6225):985.

Oliva, C., Damiens, D., Benedict, M. 2014. Male reproductive biology of *Aedes* mosquitoes. Acta Trop 132:S12-19.

Oliva, C.F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P.O., Quilici, S., ... and Boyer, S. 2012. The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized male: e49414.

O'Meara, G., Gettman, A., Evans, L.J, Curtis, G. 1993. The spread of *Aedes albopictus* in Florida. Am Entomol 39(3):163-173.

Pascini, T.V., Ramalho-Ortigão, M., Martins G.F. 2012. Morphological and morphometrical assessment of spermathecae of *Aedes aegypti* females. Memórias do Instituto Oswaldo Cruz 107(6):705-712.

Pennetier, C., Warren, B., Dabiré, K.R, Russell, I.J, Gibson G. 2010. "Singing on the wing" as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae*. Current Biology 20(2):131-136.

Perez-Staples, D., Harmer, A.M.T., Taylor, P.W. 2007. Sperm storage and utilization in female queensland fruit flies (*Bactrocera tryoni*). Physiol Entomol 32(2):127-35.

Ram, K.R., and Wolfner M.F. 2009. A network of interactions among seminal proteins underlies the long-term postmating response in *Drosophila*. Proc. Natl. Acad. Sci. USA 106:15384–89

Reidenbach, K., Cook, S., Bertone, M., Harbach, R., Wiegmann, B., Besansky, N. 2009. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. Bmc Evolutionary Biology 9(1):298.

Rogers, D., Baldini, F., Battaglia, F., Panico, M., Dell, A., Morris, H., Catteruccia, F. 2009. Transglutaminase-mediated semen coagulation controls sperm storage in the malaria mosquito. Plos Biology 7(12):e1000272.

Roth, L.M. 1948. A study of mosquito behavior - an experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). Am Midl Nat 40(2):265-352.

Sirot, L.K., Poulson, R.L., McKenna, M.C., Girnary, H., Wolfner, M.F., Harrington L.C. 2008. Identity and transfer of male reproductive gland proteins of the dengue vector mosquito, *Aedes aegypti*: potential tools.

Taylor, P.W., Kaspi, R., Yuval, B. 2000. Copula duration and sperm storage in mediterranean fruit flies from a wild population. Physiol Entomol 25(1):94-9.

Taylor, P.W., and Yuval, B. 1999. Postcopulatory sexual selection in mediterranean fruit flies: Advantages for large and protein-fed males. Anim Behav 58(2):247-54.

Twig, E., and Yuval, B. 2005. Function of multiple sperm storage organs in female mediterranean fruit flies (ceratitis capitata, diptera: Tephritidae). J Insect Physiol 51(1):67.

Ward, P.I. 2000. Cryptic female choice in the yellow dung fly *Scathophaga stercoraria* (L.). Evolution 54(5):1680-1686.

Ward, P.I. 1993. Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria* (L.). Behav Ecol Sociobiol 32(5).

Warren, B., Gibson G., and Russell I.J. 2009. Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. Current Biology, 19(6), 485-491.

Webster, D.P., Farrar, J., Rowland-Jones., S. 2009. Progress towards a dengue vaccine. Lancet Infect Dis 9: 678–687.

Young, A., and Downe, A. 1982. Renewal of sexual receptivity in mated female mosquitoes, *Aedes aegypti*. Physiol Entomol 7(4):467-71.

Chapter 1: Body Size and Spermathecal Filling

Introduction

Body size is a well-known factor that influences male mating success in a wide array of insect species. In the dipterans *Drosophila melanogaster* and *D. pseudoobscura*, larger males mated with virgin females more often than smaller males (Partridge et al., 1987). In *Anopheles freeborni,* larger males mated more often when compared to small males in swarms (Yuval et al. 1993). However copulatory success does not always influence reproductive success.

Body size in mosquitoes is proportional to the number of gametes that are stored in *Aedes* mosquitoes (Polnawat and Harrington, 2007, 2009; Renshaw et al. 1994). Larger females produce larger egg batches compared to smaller females in many species including: *Ae. cantans*, *Ae. aegypti* and *Ae. albopictus* (Renshaw et al. 1994, Farjana and Tuno 2013). Larger males of *Ae. aegypti* produced and transferred more sperm to the females reproductive tract compared to smaller males (Ponlawat and Harrington 2007, 2009).

Aedes aegypti and *Ae. albopictus* females have similar sperm storage organs. Both have a three lobed spermathecae which have one large medial lobe that is separate from a pair of 2 smaller lateral lobes (Clements and Potter 1967, Pascini et al. 2012). Typically two lobes are filled; the medial and 1 lateral. However sometimes three lobes can be filled (Clements and Potter 1967, Jones and Wheeler 1965, Pascini et al. 2012). Sperm that reach the spermathecae are used for fertilization of eggs over a female mosquito's lifetime (Clements 1999). Female *Ae. aegypti* and *Ae. albopictus* spermathecal lobes have sphincters that allow control over the entrance and exit for each lobe independently of each other (Pascini et al. 2012). In theory, this

provides females of both species the ability to select sperm from any of the three lobes to fertilize eggs.

Because large males transfer more sperm it is possible they fill more spermathecal lobes than smaller males. Males that have the ability to fill more lobes will likely increase their reproductive success. A relationship between spermathecal lobe filling and male body size has yet to be investigated. Differences in the spermathecal filling has also not been examined between *Ae. aegypti* and *Ae. albopictus*. Understanding the factors influencing spermathecal filling in both species will provide further insight into the mating and reproductive biology of these two disease vectors.

For this investigation I examined the differences in spermathecal filling of *Ae. aegypti* and *Ae. albopictus*. I compared lobe filling in mated females with small or large bodied males. I hypothesized that male body size influences spermathecal filling. I predicted that large males would fill more lobes compared to small males in both species.

Methods

Eggs for *Aedes aegypti* were previously collected in West Palm Beach, Florida and *Aedes albopictus* from Raleigh, North Carolina to establish colonies. For this set of experiments, *Ae. aegypti* were all F_6 and *Ae. albopictus* either F_2 or F_3. I hatched the *Aedes aegypti* and *Aedes albopictus* eggs in trays (Rubbermaid Egg Keeper (5.69x22.81x32.99cm), Rubbermaid, Huntersville, NC, USA) filled with 1L of tap water. Trays were placed in a 27°C incubator for 24 hours with a 14:10hr light cycle (Thermo Scientific Precision Incubator 818, ThermoScientific, Marietta, Ohio, USA). Species were hatched separately in their own trays.

Once larvae hatched I placed them into 32oz clear plastic cups with mesh lids and (Instawares Inc., Kennesaw, GA) filled with 250mL of tap water. Larvae were provided with 0.05g of finely ground coy fish food (Wardy Pond, Secaucus, NJ, USA) for both species. Trays were then placed back into the incubator. To generate large and small males I varied larval density. Small male cups contained 40 larvae and large male cups contained 25 larvae. Fifteen larval cups were made for small and large males for both species, and the larval rearing cup is the experimental unit for analysis. Additional cups for females were reared with 30 larvae each. I monitored the cups daily for the appearance of pupae.

When pupae appeared they were removed immediately from each larval cup and sorted by sex. I then placed the pupae into new 16oz cups. Each 16oz cup was associated with their larval rearing cup until they emerged as adults. Once adults emerged each cup was examined to ensure only one sex was present. Cups with both sexes were discarded.

Adults that emerged in the 16oz cups were placed in a climate controlled rearing room at 27°C with relative humidity at 80% with a 14:10 light cycle. All adults were provided with 20% sucrose solution. Four days post emergence, adults of each species were placed into 32oz cups with 5 females and 5 males from the same larval rearing cup for conspecific mating. Cups were left alone in the rearing room for 24hrs. Afterwards I removed the females and dissected out the spermathecal lobes. Females were knocked out with CO_2. Once knocked out the spermatheca was removed with insect pins under a dissection scope. After being removed, the spermathecae were picked up with a thinned out paintbrush tip and moved onto a glass slide. I then examined the spermatheca under a compound microscope at 100X to check for the presence of sperm in each lobe. Each lobe was scored as filled or not,

and a sum of the filled lobes for a given mosquito was generated (0=not inseminated, 1= one lobe, 2= two lobes, 3= all three lobes) I measured male wing lengths by the distance from the alula to the wing tip excluding any fringe scales. Wing length measurements were taken using a dissection scope and measured with a mounted camera (Olympus SXZ-LLT, Olympus Cell Sens Standard 1.7.1, MA, and USA).

A t-test was used to compare mean male wing lengths of males from "small" and "large" containers. The LOGISTIC (SAS 9.4, SAS Institute Cary, NC, USA) procedure was used to analyze lobe filling in females mated with large and small males, and between species. Lobe filling was assumed to represent an ordinal series from 0 lobes filled to all three lobes filled (0, 1, 2, 3).

Results

Average wing lengths for *Ae. aegypti* small and large males were 2.02mm and 2.17mm which significantly differed from each other (t-test, $t = -5.67$, df 26, $p < 0.001$). The average for *Ae. albopictus* small and large males was 2.08mm and 2.24mm and significantly differed from on another (t-test, $F = 2.83$, df 22, $p < 0.001$).

In females of both species the numbers of lobes filled did not differ based on male body size (GLIMMIX, df 1, 241, $F = 0.29$, $p = 0.47$). Lobe filling did not significantly differ between the two species (GLIMMIX, df 1, 241, $F = 0.52$, $p = 0.59$). *Aedes albopticus* filled all 3 three lobes more often than *Ae. aegypti*. *Aedes aegypti* filled 2 (the medial and 1 lateral) lobes 90% of the time and all three lobes 1% (Table 1.1). *Aedes albopictus* filled three lobes more often than *Ae. aegypti* (Table 1.1). *Ae. albopictus* filled 2 lobes 73% of the time (the

medial and 1 lateral lobe). Female *Ae. albopictus* filled all three lobes with small males at 11% of the time and 10% with large males (Table 1.1).

Discussion

We found that body size did not influence spermathecal lobe filling. Larger males have been shown to produce more sperm and transfer more sperm to females than smaller males in *Ae. aegypti* (Ponlawat and Harrington 2007, 2009). Body size is proportionate to the number of gametes in *Aedes* mosquito for male and female individuals (Farjan and Tuno, 2013, Polnawat and Harrington 2007, Renshaw et al. 1994). However larger males did not result in more lobes filled than small males even though previous investigations found more sperm is being deposited. Ponlawat and Harrrington (2009) counted sperm transferred to the female from the bursa and spermathecal lobes. Sperm that is transferred during copulation does not always reach the spermathecae. Jones and Wheeler, 1965 found that approximately only 62% of sperm is transferred to the spermathecae after copulation. Sperm that is not transferred to the spermathecae remains in the bursa and becomes inactive and cannot be used to fertilize eggs in *Ae. aegypti* and *Ae. albopictus* (Jones and Wheeler 1965, Olivia et al., 2014).

Female size could be the main factor in mating success and spermathecal filling. In queens of *Apis mellifera* larger females have larger spermathecae and store more sperm (Delaney et al. 2011). In the Mediterranean fruit fly larger females stored larger amounts of sperm and stored there sperm more asymmetrically when compared to small females (Taylor et al. 2000). In our investigation we did not use female size as a factor of lobe filling. Previous studies in mosquitoes have found that larger females of *Ae. aegypti* and *Ae.*

albopictus lay more eggs, which could be potentially linked to sperm storage (Farjan and Tuno 2013). Future studies should examine female size and sperm storage in the spermathecal lobes in both species.

Aedes aegypti and *Ae. albopictus* did differ in spermathecal filling. *Aedes albopictus* filled all three spermathecal lobes more often than *Ae. aegypti*. In both species it is reported that the medial lobe and one lateral lobed are filled in most matings (Jones and Wheeler, 1965, Boyer et al. 2011). Studies have not been conducted on the amount of sperm that is transferred to female *Ae. albopictus* after copulation. It is plausible that *Ae. albopictus* could transfer more sperm to females than *Ae. aegypti* males resulting in more lobes filled.

In conclusion, we found that male body size did not influence spermathecal filling in *Ae. aegypti* and *Ae. albopictus*. Behavioral differences or the amount of sperm transferred by *Ae. albopictus* males should be investigated further.

Table 1.1 Percentage of the number of lobes filled in terms of species and body size.

		Lobes Filled			
Species	**Size**	**0**	**1**	**2**	**3**
Aedes aegypti	Small	8.57	0	90.0	1.43
Aedes aegypti	Large	7.14	1.43	90.0	1.43
Aedes albopictus	Small	10.0	1.67	76.67	11.67
Aedes albopictus	Large	8.33	8.33	73.33	10.0

References

Boyer, S.J., Gilles, D., Merancienne, G., Lemperier, E., and Fontenille, D. 2011. Sexual performance of male mosquito *Aedes albopictus*. Medical and Veterinary Entomology 25 (4): 454-9

Clements, A.N., and Potter, S.A. 1967. The fine structure of the spermathecae and their ducts in the mosquito *Aedes aegypti*. J Insect Physiol 13(12):1825-1836.

Clements, A.N. 1999. The biology of mosquitoes. Wallingford, Oxon, UK ; Cambridge, MA: CABI.

Delaney, D.A., Keller, J.J., Caren, J.R.., and Tarpy, D.R. 2010. The physical, insemination, and reproductive quality of honey bee queens (*Apis mellifera* L.). Apidologie.

Jones, J.C., and Wheeler, R.E. 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. description. Biol Bull 129(1):134-50.

Oliva, C., Damiens, D., Benedict, M. 2014. Male reproductive biology of *Aedes* mosquitoes. Acta Trop 132:S12-19.

Partridge, L., Hoffmann, A., Jones, J.S. 1987. Male size and mating success in *Drosophila melanogaster* and *D. pseudoobscura* under field conditions. Elsevier B.V.

Pascini, T.V., Ramalho-Ortigão, M., Martins G.F. 2012. Morphological and morphometrical assessment of spermathecae of *Aedes aegypti* females. Memórias do Instituto Oswaldo Cruz 107(6):705-12.

Ponlawat, A., and Harrington L.C. 2009. Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. Am J Trop Med Hyg 80(3):395-400.

Ponlawat, A., and Harrington L.C. 2007. Age and body size influence male sperm capacity of the dengue vector *Aedes aegypti* (Diptera: Culicidae). J Med Entomol 44(3):422-6.

Renshaw, M., Service, M., Birley M. 1994. Size variation and reproductive success in the mosquito *Aedes cantans*. Med Vet Entomol 8(2):179-186.

Taylor, P.W., Kaspi, R.., Yuval, B. 2000. Copula duration and sperm storage in mediterranean fruit flies from a wild population. Physiol Entomol 25(1):94-9.

Young, A., and Downe, A. 1982. Renewal of sexual receptivity in mated female mosquitoes, *Aedes aegypti*. Physiol Entomol 7(4):467-71.

Yuval, B., Wekesa J.W., and Washino, R.K. 1993. Effect of body size on swarming behavior and mating success of male *Anopheles freeborni* (Diptera: Culicidae). Journal of Insect Behavior, 6(3), 333-342.

Chapter 2: Heterospecific Mating and Spermathecal Filling

Introduction

Matings between two species that are incompatible may impact reproductive success and negatively affect fitness. The negative impact of interspecific mating can go from a small cost (e.g. to a male capable of quickly and often remating) to the complete loss of fitness (e.g. for a female who is monandrous). This extreme effect on females is called satyrization and defined as sterilization that is caused by one species mating with another (Ribeiro 1988). One example of this phenomenon in nature is the heterospecific mating between *Ae. aegypti* females and *Ae. albopictus* males. Female *Ae. aegypti* that mated with A*e. albopictus* males were less likely to remate and laid eggs with unviable offspring (Leahy and Craig 1967, Tripet et al. 2011). However the reverse cross does not impact female *Ae. albopictus*. Recent studies implicate that satyrization has contributed to the displacement of naturalized populations of *Ae. aegypti* in Florida (Bargielowski et al. 2013, Tripet et al. 2011, O'Meara et al. 1995).

Multiple explanations for the displacement of *Ae. aegypti* by *Ae*. *albopictus* have been examined. Both species share similar larval habitats providing opportunities for larval resource competition (Garcia et al. 1994). *Aedes albopictus* is a more efficient competitor than *Ae. aegypti* in larval competition experiments (Edgerly et al. 1993). *Aedes albopictus* eggs were able to hatch in occupied habitats and inhibited *Ae. aegypti* egg hatching via larval density (Edgerly et al. 1993). Field studies examining larval habitats and manipulative experiments did find support for larval competition but it does not account for the rapid displacement of *Ae. aegypti* by *Ae*. *albopictus* (Juliano, 1998; Garcia et al.1994).

Competitive displacement with *Ae. albopictus* has been demonstrated in other species such as *Aedes polynesiensis, Aedes guamensis, Aedes cretinus* (Gubler 1970, Rozeboom and Bridges 1972, Giatropoulos et al. 2015). *Aedes albopictus* displaced *Ae. guamensis* decreasing its population by 95% in artificial containers in Guam and the Mariana Islands, although the ecological mechanism was unknown (Rozeboom and Bridges 1972). *Aedes albopictus* was shown to induce sterility in *Ae. polynesiensis,* suggesting satyrization may be occurring in this interaction (Gubler 1970). *Aedes albopictus* populations have recently increased in Athens, Greece (Giatropoulos et al. 2015). In a laboratory investigation Giatropoulos et al. (2015) found that *Ae. albopictus* males mated with *Ae. cretinus* females, and implied satyrization may be occurring.

Heterospecific mating and satyrization has been well documented in lab and field experiments between *Ae. aegypti and Ae. albopictus* (Nasci et al. 1989, Bargielowski et al., 2013, Bargielowski and Lounibos 2014, Tripet et al. 2009, Leahy and Craig 1967). A study conducted by Leahy and Craig (1967) investigated barriers to reproductive success in heterospecific matings between *Ae. aegypti* and *Ae. albopictus*. In their study they determined there were five barriers to reproductive success: mating behavior in relation to flight tone, structural incompatibility of genitalia, reduced oviposition, and genetic incompatibility. Another study examined the effects of male accessory gland proteins from *Ae. albopictus* males and injected them into *Ae. aegypti* females which became refractory to further mating with conspecifics (Tripet et al. 2011). The reverse cross was also conducted but *Ae. albopictus* females were unaffected (Tripet et al. 2011). However even with multiple barriers heterospecific mating still occurs.

Other investigations examined the spermathecae during heterospecific mating experiments. One study noted that, in a single case, a female *Ae. aegypti* placed *Ae. albopictus* sperm in a lateral lobe while conspecific sperm was found in the medial lobe (Tripet et al. 2011). In *Ae. albopictus* sperm of heterospecific and conspecifics were found in both medial and lateral lobes when multiple matings occurred. Inactive sperm were also detected in spermathecal lobes in cases of heterospecific mating (Leahy and Craig 1967, Nasci 1989).

Females of *Ae. aegypti* and *Ae. albopictus* both have a three lobed spermathecae with one medial lobe and a pair of smaller lateral lobes. The medial lobe is larger and has more glandular lobes which are predicted to provide sugars needed for sperm viability (Pascini et al. 2012). The lateral lobes are smaller and have fewer glandular cells (Pascini et al. 2012). In other Diptera species multiple lobed spermathecae can asymmetrically distribute sperm amongst their lobes (Ward 1993, Eberhard 1996, Fritz and Turner 2002, Fritz 2004, Twig and Yuval 2005). Females have neuromuscular control over the movement of sperm in out of the spermatheca (Wheeler and Jones 1965, Pascini et al. 2012). The importance of the asymmetrical distribution of sperm is still unclear. One potential role of multiple spermathecae maybe sperm sorting. Multiple spermathecal lobes could act as barrier to prevent incorrect fertilization by sorting sperm into different lobes for inactivation of heterospecific sperm.

In this investigation I examined if the multiple lobed spermathecae of *Ae. aegypti* and *Ae. albopictus* could sort sperm in cases of heterospecific mating. I predict that females that mated with heterospecific males will place sperm in their lateral lobes instead of the medial

lobe, reserving the medial lobe for conspecific sperm. The medial lobe is ideal for conspecific male sperm since it contains more glandular cells and can hold more sperm compared to the lateral lobes.

Methods

Eggs for *Aedes aegypti* were previously collected in West Palm Beach, Florida and *Aedes albopictus* from Raleigh, North Carolina. *Aedes aegypti* (F_6-F_8) and *Aedes albopictus* (F_3-F_5) eggs were hatched in trays (Rubbermaid Egg Keeper (5.69x22.81x32.99cm), Rubbermaid, Huntersville, NC, USA) filled with 1L of tap water. Trays were placed in a 27°C incubator for 24 hours (Thermo Scientific Precision Incubator 818, ThermoScientific, Marietta, Ohio, USA). Species were hatched separately in their own trays.

After hatching, I placed 100 larvae of both species into new trays with 1L of tap water and put back into the incubator. Each tray was given 3 pellets of coy fish food (Wardy Pond, Pellet, Secaucus , NJ, USA). I monitored trays daily for the appearance of pupae. I removed pupae from larval trays daily to prevent adult emergence. I then sorted pupae by sex. Sorted pupae were placed into 16oz cups based on sex and species with no more than 10 individuals per cup. Pupae remained in the 16oz cups till adults emerged. Once the adults emerged in the cups, I examined each cup to ensure only one sex was present. Cups with both sexes were discarded. Adults that emerged in the 16oz cups were placed in a climate controlled rearing room at 27°C with relative humidity at 80% with a 14:10 light cycle. I provided all cups and cages with adults 20% sucrose solution.

I then placed adults into 1 of 4 cages (female x male): *Aedes aegypti* x *Aedes aegypti*, *Aedes albopictus* x *Aedes albopictus*, *Aedes aegypti* x *Aedes albopictus*, *Aedes albopictus* x

Aedes aegypti with 100 adults of each sex, with 5 replicates of each mating. Cages (Bug dorm 30x30x30cm, Megaview, Taiwan) with adults were left alone for two weeks to allow ample time for mating (Bargielowski *et al*., 2013). After two weeks I removed all live females from each cage. Females were knocked down with CO_2. I then dissected out the spermathecae in each female. Spermathecae were removed with insect pins under a dissection scope. After being removed spermathecae were picked up with a thinned out paint brush tip and moved onto a glass slide. I examined the spermathecae under a light microscope at 100X to check for the presence of sperm in each lobe.

The LOGISTIC or GLIMMIX (SAS 9.4, SAS Institute Cary, NC, USA) procedure was used to analyze lobe filling in mating treatments. Lobe filling was assumed to represent an ordinal series from 0 lobes filled to all three lobes filled (0, 1, 2, 3). We also examined the pattern of lobe filling only amongst those individuals that had mated using the ordinal logistic regression (PROC LOGISTIC).

Results

Females in conspecific mating cages had a mean of 98% inseminated for both species. (Fig. 2). This was not true for heterospecific cages, in which *Ae. aegypti* females that mated with *Ae. albopictus* males had a mean insemination rate at 38% (Fig.2). The *Ae. albopictus* females and *Ae. aegypti* male cages had low insemination with a mean of 10% (Fig.2). The medial lobe and one lateral lobe were filled 90% of time in for *Ae. aegypti* and 91% for *Ae. albopictus* in conspecific matings (Table 2.1). In heterospecific matings the medial and lateral lobe were filled 27% of *Ae. aegypti* females with *Ae. albopictus* males and 7% for the reverse cross (Table 2.1). Heterospecific mating cages had many females that

were not inseminated (Table 2.1). There was a difference in numbers of lobes filled between mating conditions when individuals that did not mate were included (GLIMMIX, df 3, 1436, $F = 161.25$, $p < .0001$). When excluding individuals that did not mate there was no significant difference between mating treatments (GLIMMIX, df 3, 864, $F = 1.29$, $p = 0.27$).

Discussion

Number of lobes filled did not differ between heterospecific and conspecific mating. I predicted that females in heterospecific matings would place sperm in the lateral lobes over the medial lobe. Previous investigations found that *Ae. aegypti* females that mated with conspecific and heterospecific males stored sperm in different lobes in two cases (Tripet et al. 2011). Conspecific sperm was placed in the medial lobe and heterospecific sperm was placed in the lateral lobe (Tripet et al. 2011). For female *Ae. albopictus* sperm of conspecific and heterospecific were detected in both the medial and lateral lobe (Tripet et al. 2011).

Both *Ae. aegypti* and *Ae. albopictus* are considered to be monogamous species even though polyandry has been documented at low levels in both species (Helinski et al. 2012, Olivia et al. 2013). In our study females were placed with only one species of male. Females were therefore not given the option to differentiate sperm from multiple matings. However females were still not able to sort sperm into different lobes in heterospecific mating and filled the medial and one lateral lobe as seen in conspecific matings.

The percent insemination rates for our mating crosses were similar to previous studies conducted (Bargielowski et al. 2013). Conspecific matings insemination rates were above 90%. As seen in previous investigations, matings between *Ae. aegypti* females and *Ae. albopictus* males were more common than the reverse cross. *Aedes albopictus* and *Ae.*

aegypti may have different precopulatory behaviors. Differences in precopulatory behavior could prevent heterospecific mating. *Aedes aegypti* use harmonic convergence of wing beat frequency in mating, this has not been investigated in *Ae. albopictus* (Cator et al. 2009). The exact mechanisms of precopulatory mating in *Ae. albopictus* has been neglected and requires further inquiry.

Another study investigated allopatric and sympatric populations and found that allopatric populations were more likely to have heterospecific mating (Bargielowski et al. 2013, Bargielowski and Lounibos 2014).The strains of *Ae. aegypti* used in our study were from a sympatric population. The strain of *Ae. albopictus* probably had not been exposed to *Ae. aegypti* recently, as *Ae. aegypti* has not been found in Raleigh since the late 1980s (Harrison, personal communication).

Spermicide could be another potential role of the spermathecae in *Ae. aegypti* and *Ae. albopictus*. Previous investigations found clumped or inactivated sperm in the spermathecae of heterospecific mated females (Nasci 1989, Leahy and Craig 1967). Leahy and Craig found inactivated sperm in 85% of *Ae. aegypti* female and *Ae. albopictus* male crosses, while the reverse cross had 43% incidence of sperm clumps. Clumps or sperm were described as gelled together without movement. In a spermathecae with live sperm present, sperm is easily seen moving in a circular type motion within each spermathecal lobe. Leahy and Craig, 1967 did note that sperm in heterospecific matings were either clumped immediately after mating or were viable for several days after. Sperm clumps were also seen in the conspecific matings for *Ae. aegypti* with 5% incidence. Nasci et al. (1989) also found small amounts of dead sperm in the spermathecal lobes with matings between *Ae. aegypti* females and *Ae.*

albopictus males. During our dissections the majority of heterospecific females in both crosses contained live sperm, some also did contain dead sperm. In *Ae. aegypti* females that mated with *Ae. albopictus* males 7/249 individuals had immobile sperm present. In the reverse cross only 2/395 had dead sperm present. Our observations of immobile sperm are lower than those in previous investigations.

In conclusion we found that *Ae. aegypti* and *Ae. albopictus* do not use their spermathecal lobes for sperm sorting. Female mosquitoes in heterospecific matings still fill the medial and one lateral lobe as seen in conspecific mating. Spermicide could be alternative role which new techniques could be developed for to answer this question. The role of multiple spermathecal lobes in both species remains unknown

Table 2.1 Percentage of lobe filling outcome, N = number of females with spermathecae examined. M=medial lobe, L = lateral lobe

Crosses (female x male)	N	Empty	M only	M and 1L	2L	All
aegypti x aegypti	336	0.9	1.49	91.36	0	6.25
albopictus x albopictus	417	1.67	0.25	92.8	0	5.28
aegypti x albopictus	269	62.45	0.75	34.57	0	2.23
albopictus x aegypti	395	93.16	0.51	6.08	0	0.25

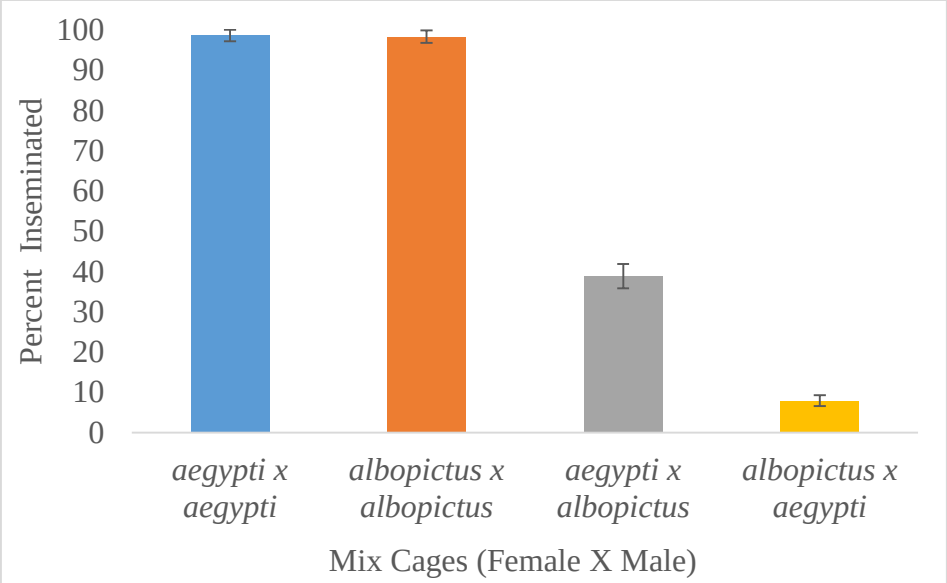

Figure 2.1 Percent of females inseminated in each type of mating cage. N = 5, Error Bars SE±.

References

Bargielowski, I., and Lounibos, L.P. 2014. Rapid evolution of reduced receptivity to interspecific mating in the dengue vector *Aedes aegypti* in response to satyrization by invasive *Aedes albopictus*. Evol Ecol 28(1):193-203.

Bargielowski, I.E., Lounibos, L.P., Carrasquilla, M.C. 2013. Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. Proc Natl Acad Sci USA 110(8):2888–2892.

Cator, L.J., Arthur, B.J., Harrington L.C., Hoy, R.R. 2009. Harmonic convergence in the love songs of the dengue vector mosquito. Science 323(5917):1077-9.

Eberhard, W.G. 1996. Female control: Sexual selection by cryptic female choice. Princeton, N.J: Princeton University Press.

Edgerly, J.S., Willey, M.S., and Livdahl, T.P. 1993. The community ecology of Aedes egg hatching: implications for a mosquito invasion. Ecological Entomology, 18(2), 123-128.

Fritz, A.H. 2004. Sperm storage patterns in singly mated females of the caribbean fruit fly, *Anastrepha suspense* (Diptera: Tephritidae). Ann Entomol Soc Am 97(6):1328-35.

Garcia, J.J., Fukuda, T., Becnel, J.J. 1994. Seasonality, prevalence and pathogenicity of the gregarine *Ascogregarina taiwanensis* (Apicomplexa: Lecudinidae) in mosquitoes from Florida. J Am Mosq Control Assoc 10(3):413–418.

Giatropoulos, A., Papachristos, D.P., Koliopoulos, G., Michaelakis, A., and Emmanouel, N. 2015. Asymmetric Mating Interference between Two Related Mosquito Species: *Aedes* (Stegomyia) *albopictus* and *Aedes* (Stegomyia) *cretinus*.

Gubler, D.J. 1970. Induced sterility in *Aedes* (Stegomyia) *polynesiensis* Marks by cross-insemination with *Aedes* (Stegomyia) *albopictus* Skuse. Journal of medical entomology, 7(1), 65-70.

Helinski, M.E., Valerio, L., Facchinelli, L., Scott, T.W., Ramsey, J., Harrington L.C. 2012. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. Am J Trop Med Hyg 86(4):635-641.

Jones, J.C., and Wheeler, R.E. 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. description. Biol Bull 129(1):134-50.

Juliano, S.A. 1998 Species introduction and replacement among mosquitoes: Interspecific resource competition or apparent competition? Ecology 79(1):255–268.

Leahy, M.G., and Craig, G.B. 1967. Barriers to hybridization between *Aedes aegypti* and *Aedes albopictus* (Diptera - Culicidae). Evolution 21(1):41-58.

Nasci, R., Hare, S., and Willis, F. 1989. Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory. Journal of the American Mosquito Control Association, 5(3), 416-421.

Oliva, C.F., Damiens, D., Vreysen, M.J., Lemperière, G., and Gilles, J. 2013. Reproductive strategies of *Aedes albopictus* (Diptera: Culicidae) and implications for the sterile insect technique.

O'Meara, G., Gettman, A., Evans, L.J., Curtis, G. 1993. The spread of *Aedes albopictus* in Florida. Am Entomol 39(3):163-173.

Pascini, T.V., Ramalho-Ortigão, M., Martin, G.F. 2012. Morphological and morphometrical assessment of spermathecae of *Aedes aegypti* females. Memórias do Instituto Oswaldo Cruz 107(6):705-12.

Ribeiro, J.M. 1988. Can satyrs control pests and vectors?. Journal of medical entomology, 25(6), 431-440.

Rozeboom, L.E., and Bridges J.R. 1972. Relative population densities of *Aedes albopictus* and *A. guamensis* on Guam. Bulletin of the World Health Organization, 46(4), 477.

Tripet, F., Lounibos, L.P., Robbins, D., Moran, J., Nishimura, N., Blosser, E.M. 2011. Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am J Trop Med Hyg 85(2):265-270.

Twig, E., and Yuva,l B. 2005. Function of multiple sperm storage organs in female mediterranean fruit flies (*Ceratitis capitata*, Diptera: Tephritidae). J Insect Physiol 51(1):67.

Ward, P.I. 1993. Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria* (L.). Behav Ecol Sociobiol 32(5).

Chapter 3: Mating Behavior

Introduction

The mating biology of *Aedes aegypti* and *Aedes albopictus* is still poorly understood even though both species are prominent disease vectors. Both species share similar behavioral characteristics: diurnal activity, container breeders, mate near a host and form small mating swarms. However differences and similarities in mating activities between these two species has yet to be thoroughly investigated. These may be important in understanding the phenomenon of displacement of *Ae. aegypti* by *Ae. albopictus* in the southeastern United States (Hobbs et al. 1991, O'Meara, 1993, Bargielowski et al. 2013).

Precopulatory behaviors can play a crucial role in reproductive success. Precopulatory behaviors have been examined in *Ae. aegypti* but not *Ae. albopictus*. Mating pairs of *Ae. aegypti* will match the harmonics of their wing beat frequency before copulation (Cator et al. 2009). The matching harmonic traits are passed down to offspring, and offspring of harmonic parents are reproductively more successful (Cator and Harrington 2011). An aggregation pheromone for *Ae. aegypti* has also been identified that attracts females to a swarm for mating (Cabrera and Jaffe 2007, Fawaz et al 2014).

Precopulatory behaviors however do not seem to prevent heterospecific mating events. Multiple investigations have found heterospecific mating between A*e. albopictus* males and *Aedes polynesiensis*, *Aedes guamensis*, *Aedes cretinus* and *Ae . aegypti* females in lab and field conditions (Tripet et al. 2011, Bargielowski et al. 2013, Nasci et al. 1989, Leahy and Craig 1967, Gubler 1970, Rozeboom and Bridges 1972, Giatropoulos et al. 2015). Species that incorrectly mate with *Ae. albopictus* have experienced declines in their populations, likely due

to satyrization (Gubler 1970, Rozeboom and Bridges 1972, Bargielowski et al. 2013). Matings between *Ae. aegypti* females with *Ae. albopictus* males have been the most well studied case of satyrization (Leahy and Craig 1967, Tripet et al. 2011, Bargielowski et al. 2013). Leahy and Craig (1967) found multiple copulation and postcopulation barriers that prevent hybridization between *Ae. aegypti* and *Ae. albopictus*. Barriers included: incompatible gentailia, genetic incompatibility, and inactivation of sperm. The precopulatory behaviors that occur before heterospecific mating have yet to be studied.

For this investigation I examined the number or mating attempts and matings over time for *Ae. aegypti* and *Ae. albopictus* mating swarms in conspecific and heterospecific mating conditions. We observed attempts and matings in small swarms to determine differences between *Ae. aegypti* and *Ae. albopictus*.

Methods

Eggs for *Aedes aegypti* were previously collected in West Palm Beach, (F_6-F_8) Florida *and Aedes albopictus* (F_3 or F_5) from Raleigh, North Carolina to establish colonies. *Aedes aegypti* and *Aedes albopictus* eggs were hatched in trays (Rubbermaid Egg Keeper (5.69x22.81x32.99cm), Rubbermaid, Huntersville, NC, USA) filled with 1L of tap water in a 27°C incubator for 24 hours (Thermo Scientific Precision Incubator 818, ThermoScientific, Marietta, Ohio, USA). Species were hatched separately in their own trays.

To generate large and small males we varied larval density. Large male trays contained 100 larvae and 250 for small. Females were reared in separate trays with 150 larvae. Each tray was given 3 pellets of coy fish food (Wardy Pond, Pellet, Secaucus, NJ, USA). Trays were monitored daily for the appearance of pupae. Pupae were removed

immediately and then separated by sex. Pupae were placed into 16oz cups. Cups with pupae were monitored daily for the emergence of adults. Adults that emerged in the 16oz cups were placed in a climate controlled rearing room at 27°C with relative humidity at 80% with a 14:10 light cycle. Adults were provided with 20% sucrose solution. Adults were then placed into new 16oz cups based on date of emergence. Adults in cups were examined before mating to prevent using contaminated individuals. Cups with both sexes were discarded.

Four-day old adults were then placed into mating cups (32oz) with 5 females and 5 males to mimic mating swarms for intraspecific and interspecific mating. Males placed in each cup were from 1 specific tray. Cups were observed for 5, 10, 15, 30 or 45 min intervals which were repeated five times each. The observer was close to the mating cups to record behavior and to provide host stimulation. The number of mating attempts (any grasp or directed flight at female), and time at which successful matings (venter to venter for 1sec, uninterrupted) (Helinski and Harrington 2012a, Oliva et al. 2013) occurred were recorded. In order for a male to successfully inseminate a female they must be in the venter to venter position (Clements 1999). Uninterrupted matings were counted since incomplete sperm transfer could account for multiple matings in a single female (Helinski et al. 2012). In *Ae. aegypti* females it takes 6sec of copulation for successful insemination, and is similar for *Ae. albopictus* (Spielman 1964, Oliva et al. 2013). The time was reduced for successful matings to 1sec since preliminary studies noted heterospecific matings were brief. After each time interval females were removed from mating cups to be dissected to confirm insemination. Females were knocked out with CO_2. Then I removed the spermathecae with an insect pins under a dissection scope. After the spermathecae was removed it was picked up with a

thinned out paintbrush tip and moved onto a glass slide. I then examined the spermathecae under a compound microscope at 100X to check for the presence of sperm in the spermatheca.

I measured male wing lengths by the distance from the alula to the wing tip excluding any fringe scales. Wing length measurements were taken with a dissection scope and measured with a mounted camera (Olympus SXZ-LLT, Olympus Cell Sens Standard 1.7.1, MA, USA).

A t-test was used to compare male wing lengths of all small and large males (SAS 9.4, SAS Institute Cary, NC, USA). To compare the number of mating attempts, the number of matings, and the number of inseminated females to the mating treatment and male body size over time, a generalized linear mixed model (GLIMMIX) was used. Distribution were checked for goodness of fit by examining the ratio of the chi-square to degrees of freedom, with values close to one suggesting a good fit. For the number of attempts, the negative binomial error distribution fit the data well (chi-square/df = 1.06). First, second and third order effects were examined, with non-significant third and second order effects removed and the model rerun. However, for the number of matings, while both the negative binomial and Poisson distribution fit well, models with second and third order terms could not converge. In this case we used a normal approximation of the data, which had a good fit (chi-square/df = 1.93) and allowed examination of interactive effects. Again, first, second and third order effects were examined, with non-significant third and second order effects removed and the model rerun.

Results

Male wing lengths for small and large reared males significantly differed from one another in all mating treatment groups (Table 3.1)

The number of attempts at mating were observed. Mating treatments did significantly differ from one another (Table 3.2). The *Ae. aegypti* female with *Ae. albopictus* male cross significantly differed from all other mating treatments (Fig 3.1). *Aedes aegypti* had the largest number of attempts compared to all other treatments (Fig. 3.1). Size was not a significant factor in number attempts overall (Table 3.2). The number of attempts observed did increase over the 45 minute observation period in all mating treatments and was significantly different (Figure 3.2) (Table 3.2).

The number of mating was also observed. Mating treatments did significantly differ from one another (Table 3.3). Conspecific mating treatments had more matings than heterospecific matings (Fig. 3.3). The number of matings that occurred increased over time (Fig. 3.4). There was no effect due to size (Table 3.3). There was a significant interaction between size and mating treatment, but subsequent corrected pairwise comparisons were not significant (all $p > 0.05$, Bonferroni correction (Table 3.3). A significant interaction was seen between mating treatment and time (Table 3.3). Over time the number of matings increase in the conspecific mating treatments, while few matings occurred in heterospecific treatments (Fig 3.4).

Some cups had females that mated more than once. In the *Ae. aegypti* and *Ae. albopictus* conspecific treatment 20% of the mating cups had more than 5 matings recorded, ranging from 6-10 observed copulations, meaning that at least one female of the five mated

more than once. Multiple matings of females were seen in both small and large male cups. Females in heterospecific cups did not have multiple matings.

Discussion

In our study we examined the timing of mating interactions in conspecific and heterospecific mating for *Ae. aegypti* and *Ae. albopictus*. We found that the number of attempts significantly differed over time and mating treatment. The number of attempts increased with time. In conspecific mating there were more attempts made than heterospecific mating. Other studies examining heterospecific mating recorded similar results (Bargielowski and Lounibos 2014, Tripet et al. 2011, Leahy and Craig 1967). The same trends were seen for the number of matings as well, in which there were more matings between conspecifics than heterospecifics. There was a significant interaction between mating treatment and time as well.

In our study we attempted to create ideal conditions to observe both conspecific and heterospecific mating. Conditions worked well for *Ae. aegypti* conspecific matings that had the greatest number of attempts and matings observed. *Aedes albopictus* did not mate as often as *Ae. aegypti* but other factors may have influenced those results. In the heterospecific mating treatment few matings were observed.

To create ideal conditions, I implemented multiple tactics to mimic natural mating settings in the lab. Mating in both *Ae. aegypti* and *Ae. albopictus* typically occur near a host or in small swarms. Many studies on mosquito mating biology have been conducted without host stimulation (Bargielowski and Lounibos 2014, Polnawat and Harrington 2009, Leahy and Craig 1967). However during observations the observer was close to cup providing host

stimulation. In this study I attempted to reproduce a small swarm by placing 5 males and females in each cup. Swarms were used in this study since in field settings mating swarms increased copulation frequency (Cabrera and Jaffe 2007). Cups had a limited amount of space compared to field conditions so numbers of individuals in the swarms were reduced. Density of mosquito mating cages can impact the number of matings that occur and can lead to artificially high insemination rates (Ponlawat and Harrington 2009). Having the higher frequency setting was ideal for heterospecific matings since they do not occur readily in the field or in lab settings. However the reduced swarm size may have not had enough individuals. In field studies swarms of *Ae. aegypti* had on average 23 individuals and 3- 40 individuals for *Ae*. *albopictus* (Cabrera and Jaffe 2007, Gubler and Bhattacharya 1972).

Mosquito swarms were presented with 5 to 45 minutes to mate within the cups. In *Ae. albopictus* swarms in field conditions the highest frequency of matings occurred between 5 to 10 minutes (Gubler and Bhattacharya 1972). In studies previously conducted with heterospecific mating, small swarms were given 15 minutes to mate (Bargielowski and Lounibos 2014). Bargielowski and Lounibos (2014) found *Ae. aegypti* females were inseminated by *Ae. albopictus* males 32-61% of time after 15 minutes of exposure in strains not resistant to satyrization. Based upon previous studies, the mosquitoes were provided sufficient amount of time for mating between conspecifics and heterospecifics.

Aedes aegypti females have been shown to more likely mate with *Ae. albopictus* than the reverse cross (Bargielowski et al. 2013, Leahy and Craig 1967, Nasci et al. 1989, De Jesus, 2015). In our study we did not see any significant differences between the heterospecific mating treatments. Factors including space within in the cups and time might

have interfered. Virgin male and female mosquitoes were placed in mating treatment cups 4 days after emergence. Peak mating occurs 3-5 days after emergence (Leahy and Craig 1967). By using 4 day old male and females we expected mating activity in our mating treatments. *Aedes aegypti* and *Ae. albopictus* males take approximately 24 hours for male terminila to rotate and once this is complete males can mate (Roth 1948, Oliva et al. 2012). Since males were exposed to females well after this time period, males should have been capable of mating. In other studies mosquito pairs were stimulated to mate by tilting a tube back a forth (Oliva et al. 2013). My cups were not shaken or disturbed in any form during observations which could have influenced the number of matings observed across all treatments.

In our study we wanted to observe the interactions leading up to heterospecific mating between *Ae. aegypti* and *Ae. albopictus*. A previous study found that allopatric and sympatric populations of *Ae. aegypti* responded to the presence of *Ae. albopictus* differently (Bargielowshi and Lounibos 2014). Sympatric strains of *Ae. aegypti* were less likely to mate with *Ae. albopictus* than allopatric strains (Bargielowski et al. 2013). The strain of *Ae. aegypti* used in our study was from West Palm Beach Florida and therefore likely to have had a history of exposure to *Ae. albopictus* (Reiskind and Lounibos 2013). Another study found that over 1-3 generations of *Ae. aegypti* exposed to *Ae. albopictus* became resistant to satyrization and *Ae. aegypti* that became resistant to satyrization took longer to mate with conspecifics (Bargielowski and Lounibos 2014). However, this artificially selected avoidance of heterospecific mating is also lost over 5 generations in the absence of exposure to heterospecifics (Bargielowski and Lounibos, unpublished data). Because our *Ae. aegypti* population had been kept away from *Ae. albopictus* for at least six generations, we expected

that they would respond similarly as allopatric populations in the heterospecific mating treatment. However low amounts of mating and insemination in the heterospecific mating cups were observed in our study. The factors previously discussed may have played a factor in the low levels of mating observed, and the 45 minute exposure period might be insufficient in heterospecific matings.

A few incidences of polyandry were seen in our mating cups. In the conspecific treatments 20% of the cups had cases of polyandry which is similar to previous investigations. In a study with *Ae. aegypti* in semi-field conditions 14% of had mated more than once (Helinski et al. 2012c). For *Ae. albopictus*, approximately 26% of females collected from the field had progeny from than one male (Boyer et al. 2013). In spite of this empirical data, both species are typically considered monandrous (Clements 1999). Seminal protein fluids (Spfs) alter female behavior after copulation preventing females from mating again for multiple gonotrophic cycles (Helinski et al. 2012a). In heterospecific matings Spfs of *Ae. albopictus* males cause the satyrization of *Ae. aegypti* females, by rendering them refractory to further matings and may occur even in the absence of sperm deposition (Oliva et al. 2013). Using cues for mating in precopulatory behavior is crucial for *Ae. aegypti* females. How *Ae. aegypti* confuses precopulatory signals or why *Ae. albopictus* males do not recognize correct females is still unknown.

Other studies have investigated the interactions between these two species during their adult stages. One study examined feeding harassment in cages with females placed with conspecific males or heterospecific. *Aedes albopictus* was not impacted conspecific or heterospecific males during feeding (Soghigian et al. 2014). However in *Ae. aegypti*

heterospecific males reduced feeding occurrence (Soghigian et al. 2014). In another harassment study conducted with *Ae. aegypti* harassment by males did not suffer a fitness cost (Helinski and Harrington 2012b).

Precopulatory barriers between species should be further examined. Visual, auditory and pheromones should be compared between these two species. Flight tone harmonics and pheromones have proven to play a crucial roles in *Ae. aegypti* and could also be important *Ae. albopictus* mating and cross mating between the two species. Understanding the differences between these species is important to the development of new vector control techniques to help prevent disease transmission.

Table 3.1 Average male wing length (mm) for mating treatment. P-values determine from t-tests (N =50, df =48) between small and large males used in mating cups.

Mating Treatment	Small	Large	p-value
Conspecific *Ae. aegypti*	1.84	2.12	>.0001
Conspecific *Ae.albopictus*	1.82	2.06	>.0001
Heterospecific *Ae. aegypti*	1.82	1.95	0.0009
Heterospecific *Ae. albopictus*	1.78	1.95	>.0001

Table 3.2 PROC GLIMMIX table for the number of attempts, using a generalized linear mixed model with a negative binomial distribution. MT = mating treatment

EFFECT	Num DF	Den DF	F Value	Pr > F
MT	3	183	18.43	<.0001
SIZE	1	183	0.44	0.5062
MT*SIZE	3	183	1.52	0.2110
TIME	4	183	36.57	<.0001

Table 3.3 PROC GLIMMIX table for the number of matings, using a generalized linear mixed model with a normal approximation. MT= Mating treatment.

EFFECT	Num DF	Den DF	F Value	Pr > F
MT	3	174	22.52	<.0001
SIZE	1	174	0.98	0.3248
TIME	4	174	11.63	0.0289
MT*TIME	12	174	2.69	<.0001

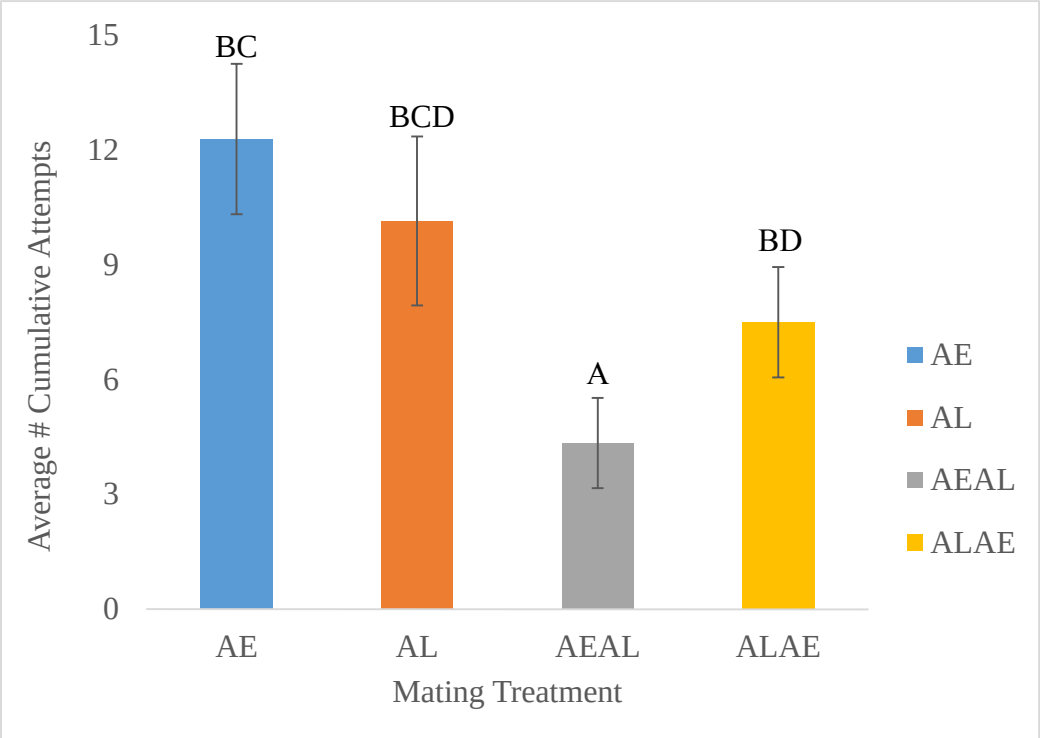

Figure 3.1 Mean number of attempts for each mating treatment. *Ae. aegypti* (AE), *Ae. albopictus* (AL), AEAL and ALAE (Female x Male crosses). Letters are homogenized groups from bonferroni correction. Error bars = SE±

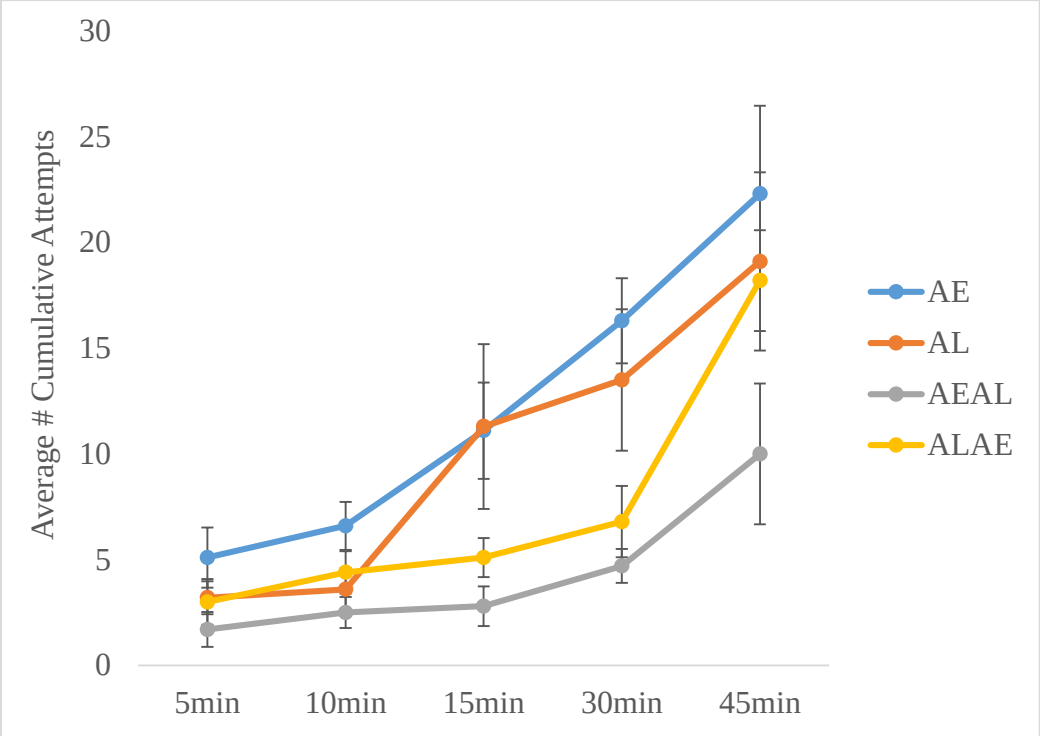

Figure 3.2 The average cumulative number of mating attempts over time. AE = *Ae. aegypti*, AL= *Ae. albopictus*, AEAL and ALAE (Female x Male crosses). Error bars = SE±

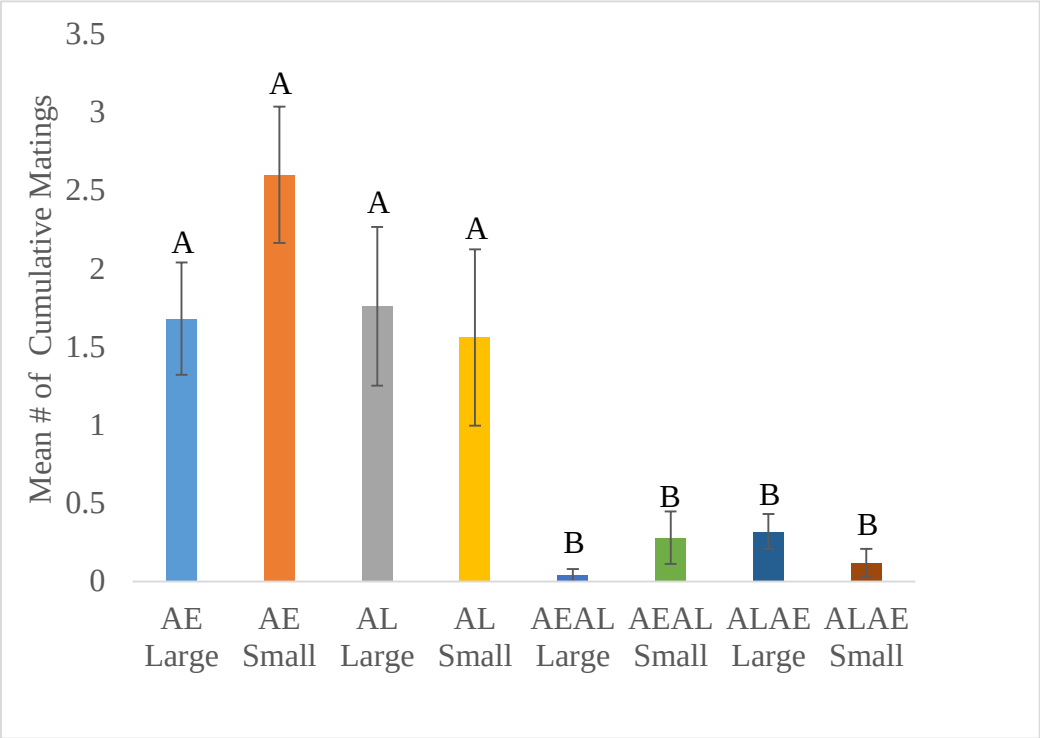

Figure 3.3 Mean number of matings for each mating treatment. *Ae. aegypti* (AE), *Ae. albopictus* (AL), AEAL and ALAE (Female x Male crosses). Letters are homogenized groups from bonferroni correction. Error bars = SE±

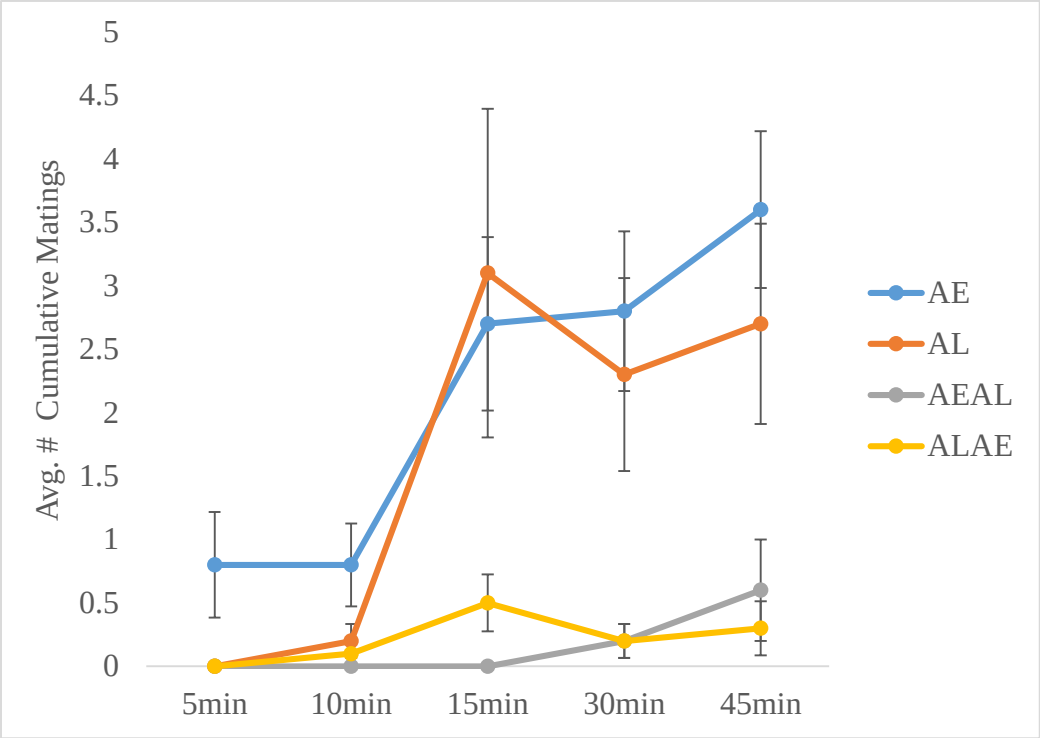

Figure 3.4 Average number of cumulative matings over time for each mating treatment. *Ae. aegypti* (AE), *Ae. albopictus* (AL), AEAL and ALAE (Female x Male crosses). Error bars = SE±

References

Bargielowski, I., and Lounibos, L.P. 2014. Rapid evolution of reduced receptivity to interspecific mating in the dengue vector aedes aegypti in response to satyrization by invasive *Aedes albopictus*. Evol Ecol 28(1):193-203.

Bargielowski, I.E., Lounibos, L.P., Carrasquilla, M.C. 2013. Evolution of resistance to satyrization through reproductive character displacement in populations of invasive dengue vectors. Proc Natl Acad Sci USA 110(8):2888–2892.

Boyer, S., Toty, C., Jacquet, M., Lempérière, G., Fontenille, D. 2012. Evidence of multiple inseminations in the field in *Aedes albopictus*. PloS One 7(8):e42040.

Cabrera, M., and Jaffe, K. 2007. An aggregation pheromone modulates lekking behavior in the vector mosquito *Aedes aegypti* (Diptera: Culicidae). J. Am. Mosq. ControlAssoc. 23, 1–10.

Cator, L.J., and Harrington, L.C. 2011. The harmonic convergence of fathers predicts the mating success of sons in *Aedes aegypti*. Anim Behav 82(4):627-633.

Cator, L.J, Arthur, B.J., Harrington, L.C., Hoy, R.R. 2009. Harmonic convergence in the love songs of the dengue vector mosquito. Science 323(5917):1077-1079.

Clements, A.N. 1999. The biology of mosquitoes. Wallingford, Oxon, UK ; Cambridge, MA: CABI.

Giatropoulos, A., Papachristos, D.P., Koliopoulos, G., Michaelakis, A., and Emmanouel, N. 2015. Asymmetric Mating Interference between Two Related Mosquito Species: *Aedes* (Stegomyia) *albopictus* and *Aedes* (Stegomyia) *cretinus*.

Gubler, D.J. 1970. Induced sterility in *Aedes* (Stegomyia) *polynesiensis* Marks by cross-insemination with *Aedes* (Stegomyia) *albopictus* Skuse. Journal of medical entomology, 7(1), 65-70.

Gubler, D.J., and Bhattacharya, N.C. 1972. Swarming and mating of *Aedes*-(*S*)-*albopictus* in nature. Mosquito News 32(2):219-223.

Helinski, M.E., Deewatthanawong, P., Sirot, L.K., Wolfner, M.F., Harrington, L.C. 2012a. Duration and dose-dependency of female sexual receptivity responses to seminal fluid proteins in *Aedes albopictus* and *Ae. aegypti* mosquitoes. J Insect Physiol 58(10):1307-1313.

Helinski, M.E., and Harrington, L.C. 2012b. The role of male harassment on female fitness for the dengue vector mosquito *Aedes aegypti*. Behavioral ecology and sociobiology, 66(8), 1131-1140.

Helinski, M.E., Valerio, L., Facchinelli, L., Scott, T.W., Ramsey, J., Harrington, L.C. 2012c. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. Am J Trop Med Hyg 86(4):635-641.

Hobbs, J., Hughes, E., Eichold, B. 1991. Replacement of *Aedes-aegypti* by *Aedes-albopictus* in Mobile, Alabama. J Am Mosq Control Assoc 7(3):488-489.

Leahy, M.G., and Craig, G.B. 1967. Barriers to hybridization between *Aedes aegypti* and *Aedes albopictus* (Diptera - Culicidae). Evolution 21(1):41-58.

Nasci, R., Hare, S., and Willis, F. 1989. Interspecific mating between Louisiana strains of *Aedes albopictus* and *Aedes aegypti* in the field and laboratory. Journal of the American Mosquito Control Association, 5(3), 416-421.

Oliva, C.F., Damiens, D., Vreysen, M.J., Lemperiere, G., Gilles, J. 2013. Reproductive strategies of *Aedes albopictus* (Diptera: Culicidae) and implications for the sterile insect technique: E78884. Plos One 8(11).

O'Meara, G., Gettman, A., Evans, L.J., Curtis, G. 1993. The spread of *Aedes albopictus* in Florida. Am Entomol 39(3):163-173.

Ponlawat, A., and Harrington, L.C. 2009. Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. Am J Trop Med Hyg 80(3):395-400.

Reiskind, M.H., and Lounibos, L.P. 2013. Spatial and temporal patterns of abundance of *Aedes aegypti* L. (*Stegomyia aegypti*) and *Aedes albopictus* (Skuse) [*Stegomyia albopictus* (Skuse)] in southern Florida. Medical and veterinary entomology, 27(4), 421-429.

Roth, L.M. 1948. A study of mosquito behavior - an experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). Am Midl Nat 40(2):265-352.

Rozeboom, L.E., and Bridges, J.R. 1972. Relative population densities of *Aedes albopictus* and *A. guamensis* on Guam. Bulletin of the World Health Organization, 46(4), 477.

Spielman, A. 1964. The mechanics of copulation in *Aedes aegypti*. The Biological Bulletin, 127(2), 324-344.

Soghigian, J., Gibb,s K., Stanton, A., Kaise,r R., and Livdahl, T. 2014. Sexual Harassment and Feeding Inhibition Between Two Invasive Dengue Vectors. Environmental health insights, 8(Suppl 2), 61.

Tripet, F., Lounibos, L.P., Robbins, D., Moran, J., Nishimura, N., Blosser, E.M. 2011. Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am J Trop Med Hyg 85(2):265-270.

Chapter 4: Sperm Depletion

Introduction

Multiple sperm storage organs are found in a variety of Diptera species (Eberhard 1996). Dipterans typically have 2-4 spermathecal lobes (Eberhard 1996). Lobe filling and depletion is dependent on muscular control of the female reproductive tract in dipterans long after mating (Linley and Simmons 1981). In Diptera species with multiple lobed spermathecae, some distribute their sperm asymmetrically across multiple lobes (Eberhard 1996). Examples lobes include: *Scathophaga stercoraria* (yellow dung fly), *Anastrepha suspense* (Caribbean fruit fly), *Bactrocera tryoni* (Queensland fruit fly), *Aedes aegypti* and *Aedes albopictus* (Ward, 1993; Fritz and Turner 2002, Perez et al. 2007, Clements 1999). The usage of multiple spermathecal lobes has been well investigated in the yellow dung fly. Previous studies found that female young dung flies that mated with larger males stored sperm in the paired spermatheca (Ward 1993). Females that mated with larger males also fertilized more eggs than smaller males, who stored sperm in the singlet lobe (Ward 1993). In *Ae. aegypti* and *Ae. albopictus* we found that body size did influence spermathecal filling but did examine lobe usage (De Jesus 2015).

Previous investigations have yet to address the usage of multiple lobe spermatheca in mosquitoes. *Aedes aegypti* and *Ae. albopictus* both have a three lobed spermathecae, with a larger medial lobe and paired smaller lateral lobes, which is similar to that of the yellow dung fly. Females of *Ae. aegypti* are capable of controlling the entrance and exit of sperm from each individual lobe (Pascini et al. 2012). In *Ae. aegypti* and *Ae. albopictus* females typically fill the medial and one of the lateral lobes but rarely all three lobes (Jones and Wheeler 1965,

Pascini et al. 2012, Olivia et al. 2013, De Jesus 2015). In *Ae. aeypti* the medial lobe has more glandular cells than the lateral lobe that provide sugars for sperm viability (Pascini et al. 2012). Female mosquitoes fertilize their eggs immediately before oviposition (Clements 1999). However which lobes are used in egg fertilization has not been examined in *Ae. aegypti* and *Ae. albopictus*. In this investigation we examined females that mated with large and small males and counted the number of sperm present after multiple gonotrophic cycles. I hypothesize that females select sperm from their medial lobe over the lateral lobe. I also observed the number of eggs laid over each gonotrophic cycle and the longevity of females.

Methods

Eggs for *Aedes aegypti* were previously collected in West Palm Beach, (F_8) Florida *and Aedes albopictus* (F_5) from Raleigh, North Carolina. *Aedes aegypti* and *Aedes albopictus* eggs were hatched in trays (Rubbermaid Egg Keeper (5.69x22.81x32.99cm), Rubbermaid, Huntersville, NC, USA) filled with 1L of tap water in a 27°C incubator for 24 hours (Thermo Scientific Precision Incubator 818, ThermoScientific, Marietta, Ohio, USA). Species were hatched separately in their own trays.

Trays had varying larval density to produce different male body sizes. Large male trays contained 100 larvae and 250 for small. Females were reared in separate trays with 150 larvae. Each tray was given 3 pellets of coy fish food (Wardy Pond, Pellet, Secaucus, NJ, USA). Trays were monitored daily for the appearance of pupae. Pupae were removed immediately and then separated by sex and size. Pupae were placed into 16oz cups. Cups with pupae were monitored daily for the emergence of adults. Adults that emerged in the 16oz cups were placed in a climate controlled rearing room at 27°C with relative humidity at

80% with a 14:10 light cycle. Adults were provided with 20% sucrose solution. Adults in cups were examined before mating to prevent using contaminated individuals. Cups with both sexes were discarded.

Females were placed in cages (Bug dorm 30x30x30cm, Megaview, Taiwan) with 100 large or small conspecific males. Females were left to mate with males for 48hrs. Females were then blood feed on a human volunteer and placed into 16oz cups with a small 25mL cup placed at the bottom. The small cup was filled with 15mL of tap water. A piece of seed paper was wrapped around the edge of the small cup for egg laying. Females were given 1 week to lay eggs before they were offered blood again. Egg papers and water were changed between each cycle. Females were kept alive for 1-4 gonotropic cycles. After each cycle 10 live females were sacrificed to examine the amount of sperm present in each spermathecal lobe.

Live females were knocked out with CO_2 before dissections and the spermatheca was dissected. The spermathecae were picked up with a thinned out paint brush tip and rinsed in PBS. The 3 spermathecal lobes were then separated from one another. If lobes were torn during the dissection process, those female samples were discarded. Each individual lobe was then placed on to its own glass slide in 7uL of PBS. Lobes were then torn apart with insect pins till a sperm clump was no longer present, as observed under a phase contrast microscope. Dead females were not used for dissections since sperm in the lobes became clumped and could not be dispersed well enough for accurate sperm counts. An 18x18mm coverslip was then placed on the slide. Slides were then dried for 24hrs and then examined under a phase contrast microscope (200x) and the number of sperm was counted.

Males and females had wing lengths measured which was determined by the distance from the alula to the wing tip. Wing length measurements were taken using a dissection scope and measured with a mounted camera (Olympus SXZ-LLT, Olympus Cell Sens Standard 1.7.1, MA, and USA).

A t-test was used to compare male wing lengths of all small and large males (Proc t-test, SAS 9.4, SAS Institute Cary, NC, USA). I used a linear mixed model to determine the effects of male size, species, and gonotrophic cycle on sperm count (total and medial and lateral separately), female survival (only females that died naturally, not those sacrificed for sperm counts), and egg production, while accounting for female wing length as a covariate (Proc GLM, SAS 9.4, SAS Institute, Cary, NC, USA). The second lateral lobe was not examined since none of the *Ae. aegypti* filled it and only 4 *Ae. albopictus* did fill it. For egg production, we examined eggs produced in each gonotrophic cycle, and then mean eggs per gonotrophic cycle to compare individual females that died or were sacrificed at different times. Because of a well-established relationship between fecundity and female wing length in mosquitoes, we retained female wing length as a covariate in all our models. For longevity of females, the model compared male body size and species, with wing length as a covariate.

Results

Large and small males were reared to determine if body size influenced sperm usage in the spermathecal lobes. Large and small males for both *Ae. aegypti* (t-test, df 198, t =-12.39, $p < 0.001$) and *Ae. albopictus* (t-test, df 184, t= -7.24, $p < 0.001$) significantly differed from one another. The average wing length for *Ae. aegypti* large males was 2.01 and small males 1.77mm. The average wing length for *Ae. albopictus* small males was 1.97mm and

large males 2.14mm. The amount of sperm was examined in each spermathecal lobe to determine lobe usage in *Ae. aegypti* and *Ae. albopictus*. *Aedes albopictus* stored more sperm in total compared to *Ae. aegypti* throughout all cycles (GLM, df 5,157, F = 5.21, p =0.0002) (Fig 4.2). The medial lobe had significantly more sperm in *Ae. albopictus* than *Ae. aegypti* over all gonotrophic cycles (GLM, df 5, 157, F = 8.06, p = <.0001) There were no significant differences between species when examining sperm counts in the lateral lobe (GLM, df 5,157, F = 0.98, p = 0.43).

In *Ae. aegypti* sperm decreased in both the medial and lateral lobe over gonotrophic cycles, with a maximum mean decline of 84 sperm from the medial and 42 sperm from the lateral lobes (medial: GLM, df 4,82, F = 6.98, p = 0.0006; lateral: GLM, df 4, 82, F = 2.53, p = 0.0467) (Figs 4.3, 4.4). In *Ae. albopictus* there were no significant differences in sperm counts over multiple gonotrophic cycles for the medial (GLM, df 4,71, F = 0.36, p = 0.838)(Fig 4.5) and lateral lobe (GLM, df 4,71, F = 1.89 , p = 0.122) (Fig 4.6).

Aedes aegypti that mated with small males but died naturally lived 21 days on average while those that mated with large males lived for 22 days on average. For *Ae. albopictus* that died naturally those that mated with small males lived on average 18 days while those that mated with large males averaged 20 days. These differences were insignificant (p>0.05).

In both *Ae. aegypti* and *Ae. albopictus* male size was a significant factor in egg production over gonotrophic cycles 1-3 but not in gonotrophic cycle 4 (Table 4.1). Females of both species that mated with larger males laid more eggs compared to females mated with smaller males over multiple gonotrophic cycles (Fig. 4.1). *Aedes albopictus* that mated with

either small or large males laid more eggs than *Ae. aegypti* over all cycles expect in cycle 3, where *Ae. aegypti* females that mated with large males laid more eggs than *Ae. albopictus* females that mated with small males with female wing length as a covariate in the model. *Aedes albopictus* (2.28mm) females were also significantly larger than *Ae. aegypti* (2.04mm) females based on wing length measurement (t-test, df 198, t-stat -9.08, $p < 0.0001$).

Discussion

In this investigation I examined the amount of sperm present in the spermathecal lobes to determine if females were preferentially using sperm from the medial or lateral lobes. I counted sperm in each lobe from different individuals over multiple gonotrophic cycles. Lastly we counted the number of eggs laid for each female after each gonotrophic cycle.

From *Ae. aegypti*, I was able to detect changes in the number of sperm over time in each lobe but without clear indication which lobe sperm was being selected from, with significantly lower sperm counts in the third and fourth gonotrophic cycles. While there was fluctuation in sperm count from cycle to cycle in *Ae. albopictus*, there was no clear pattern of depletion from either lobe. Detection of sperm depletion could be difficult for several reasons. Females of both species in our study laid anywhere from 10-78 eggs/gonotrophic cycle, with the most fecund female laying 231 eggs over four gonotrophic cycles. The medial lobe is calculated to hold 660 sperm while the lateral can hold up to 486, for a total of around 1100 sperm (Jones and Wheeler, 1965; Linley and Simmons, 1981). With an estimated 1:1 sperm to egg ratio theory it may be difficult to detect the 10-20% changes in sperm number within the

spermathecal lobes without the most precise counts considering the variation between individuals in the total number of sperm counted.

In addition, my methodology could have influenced sperm counts. I selected to pop spermathecal lobes directly on the slide get the most direct count of sperm. I mixed the sample with insect pins on the slide which could account for an increase in error. Other staining techniques have been used to count mosquito sperm but require mixing of stains and transferring of sperm through multiple containers which could be a source of even greater losses of sperm. Variation is likely to occur in any of these methods which could account for not detecting lobe selection. Similar methodologies to our study had been used to conducted sperm counts in other dipteran species (Perez et al., 2007; Yuval et al., 1996).

Other dipteran species have displayed the ability to select sperm from different spermathecal lobes. The asymmetrical usage of spermathecal lobes has been well documented in the yellow dung fly (*Scathophaga stercoraria*) (Ward 1993, 2000). However the yellow dung fly is a polyandrous species while *Ae. aegypti* and *Ae. albopictus* are considered monandrous (Clements 1999). Mosquitoes may have not displayed asymmetrical usage of spermathecal lobes since females only mate once and therefore there is not a need for post-copulatory selection. Polyandry does occur in both species at low frequencies (10-14%) as seen in lab and field conditions (Helinski et al. 2012, Boyer et al. 2012). In our study it is plausible that some females could have mated more than once in the mating cages but we did not test females for the presence of sperm from multiple males. Females are likely to mate more than once if they interrupted during mating or mate with a sperm depleted male (Gwadz and Craig 1970). The cages we used had high densities to produce large numbers of inseminated females.

However high density cages have been shown to increase insemination rate and increase the number of interrupted matings (Polnawat and Harrington 2009, Roth 1948). If females mated with multiple males only 2/3 of the sperm transferred to female are transported to the spermathecae after copulation (Jones and Wheeler 1965). It is therefore unlikely that multiple matings would influence lobe selection.

In our study we did not find any differences in the longevity of *Ae. aegypti* or *Ae. albopictus* that mated with small or large males. Female longevity in *Ae. aegypti* has been linked to seminal protein fluids transferred during copulation (Helinski and Harringtion 2011). It was found that female *Ae aegypti* that mated with virgin males had greater longevity than females that mated with non-virgin males but it is unknown if this occurs in *Ae. albopictus* (Helinski and Harrington 2011). It is possible that male body size might influence female longevity, but our study design, which did not intend to compare longevity, was unable to detect that effect.

In our investigation we did find significant differences in egg laying in *Ae. aegypti* and *Ae. albopictus*. We found that *Ae. albopictus* typically laid more eggs than *Ae. aegypti* across different body sizes of male and female wing lengths. In a study by Klowden and Chambers (1992), *Ae. albopictus* had a greater reproductive capacity than *Ae. aegypti*. They found that *Ae. albopictus* accumulates more lipids and glycogen during its longer larval development time compared to *Ae. aegypti* and laid more eggs. Because *Ae. albopictus* had access to greater amount of resources they were able to lay eggs with small blood meals compared to *Ae. aegypti* (Klowden and Chambers, 1992). In our study females were allowed to blood feed until they were fully engorged giving all females similar resources. In Klowden and Chambers, 1992

both species had similar protein content as adults, however *Ae. aegypti* eggs had greater amount of proteins and lipids than *Ae. albopictus* which could reduce the number of eggs laid. Previous studies also found that the number of eggs laid in female *Ae. aegypti* and *Ae. albopictus* is influenced by body size of the female (Farjan and Tuno, 2013; Renshaw et al., 1994). Larger females (based on wing length) of laid more eggs than smaller females in *Ae. aegypti*, *Ae. albopictus* and *Ae. cantans*, a result I also observed as a female wing length was a significant covariate in this experiment (Farjan and Tuno, 2013; Renshaw et al., 1994).

Male size significantly increased fecundity in both species. The effect of male size on female fecundity in insects is unclear, with some studies reporting negative and others reporting positive impacts. In *Drosophila melanogaster* females that mated with larger males had decreased longevity and fecundity (Pitnick and García–González, 2002). However in the butterfly *Pieris napi* femlaes that mated with large males benefited since they obtained nutrients from the male ejaculate which resulted in increased fecundity (Wiklund and Kaitala, 1995). One male-related factor that could have influenced the number of eggs laid could be due to seminal protein fluids (Spfs). Seminal protein fluids have been found to increase egg production or stimulate ovulation in multiple dipteran species (Avila et al. 2011). In *D. melanogaster* 4 Spfs stimulate egg laying in mated females (Gillott, 2003; Ram and Wolfner, 2007). In *Ae. aegypti* the Spfs increase oviposition, but it is unknown if this occurs in *Ae. albopictus* as well (Gillott, 2003; Sirot et al., 2008). Larger males may transfer more Spfs during copulation which could result in a greater number of eggs laid.

In conclusion we were unable to determine conclusively if *Ae. aegypti* and *Ae. albopictus* select sperm from different spermathecal lobes. We were still able to see a decrease

in the amount of sperm over multiple cycles in *Ae. aegypti* but not in *Ae. albopictus*. We found no effect of male size on female longevity. We did find that *Ae. albopictus* laid more eggs than *Ae. aegypti* which supports previous research (Klowden and Chambers, 1992). In both species, females that mated with larger males laid more eggs than those that mated with smaller males, although we do not know the mechanism for this increase in fecundity. This investigation is one a few studies conducted examining the usage of multiple spermathecal lobes and their role in fertilization. It is also the first to address this topic in mosquitoes. Overall this study provides valuable insight on the reproductive biology of *Ae. aegypti* and *Ae. albopictus* that could be applied in developing new forms of vector control.

Table 4.1 Factors for the ANOVA for each gonotrophic cycle and mean eggs from in each cycle. WL = Wing Length

Cycle	Factor	F Value	DF	Pr > F	Egg Mean
1	**Size**	8.18	1	0.0048	35.12
	Species	12.46	1	0.0005	
	Size*Speceis	7.51	1	0.0068	
	WL	8.74	1	0.0036	
2	**Size**	7.43	1	0.0076	33.9
	Species	22.59	1	<.0001	
	Size*Speceis	1.21	1	0.2748	
	WL	0.83	1	0.3654	
3	**Size**	4.22	1	0.045	37.97
	Species	1.35	1	0.2511	
	Size*Speceis	0.03	1	0.8551	
	WL	0.41	1	0.5256	
4	**Size**	4.12	1	0.0559	37.88
	Species	16.7	1	0.0006	
	Size*Speceis	4.81	1	0.0403	
	WL	0.05	1	0.8277	

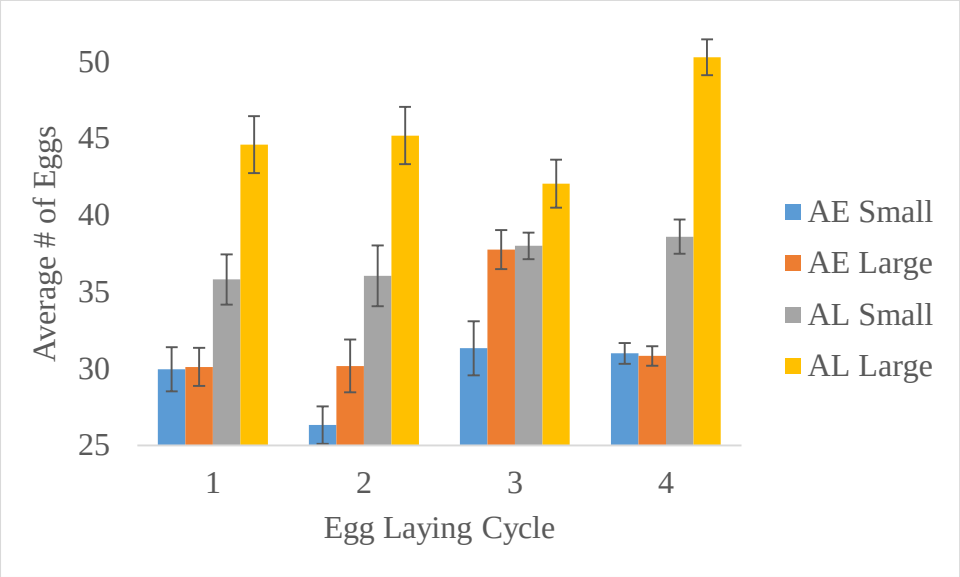

Figure 4.1 The average number of eggs laid in each cycle for *Ae. aegypti* (AE) and *Ae. albopictus* (AL) females that mated with small and large males. SE = ±

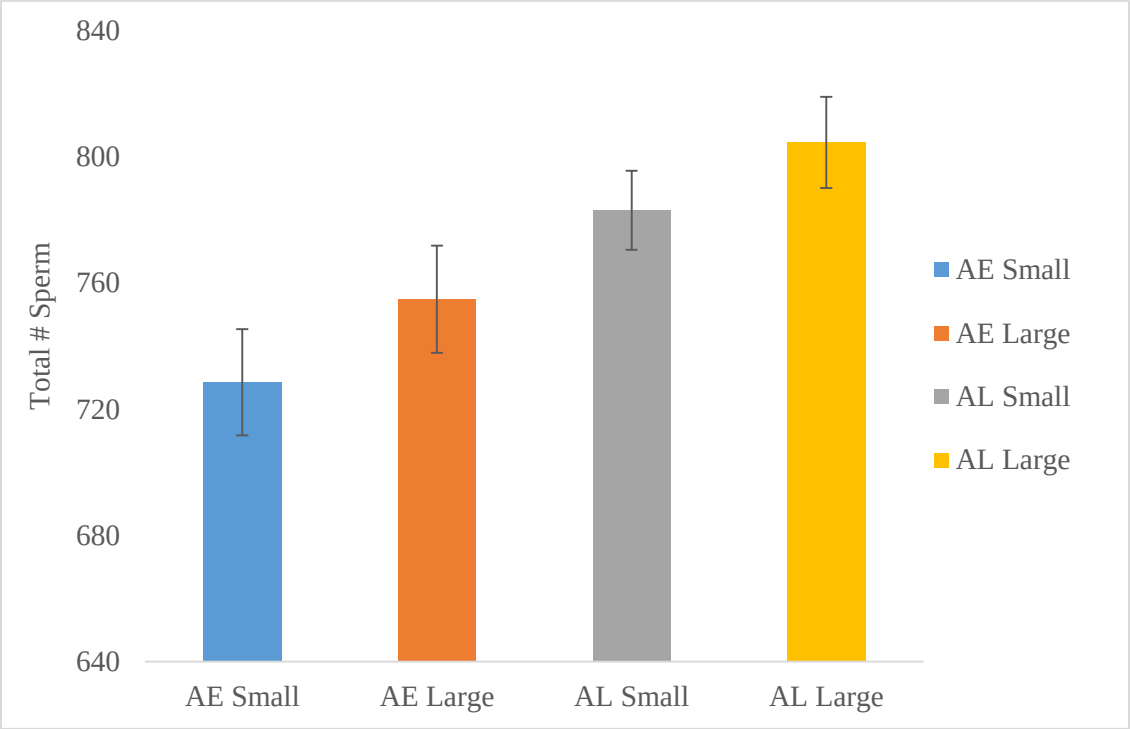

Figure 4.2 The average total number or sperm present in both the medial and lateral lobe for *Ae. aegypti* and *Ae. albopictus* over multiple gonotrophic cycles. The lines connect the average total of sperm in each cycle.

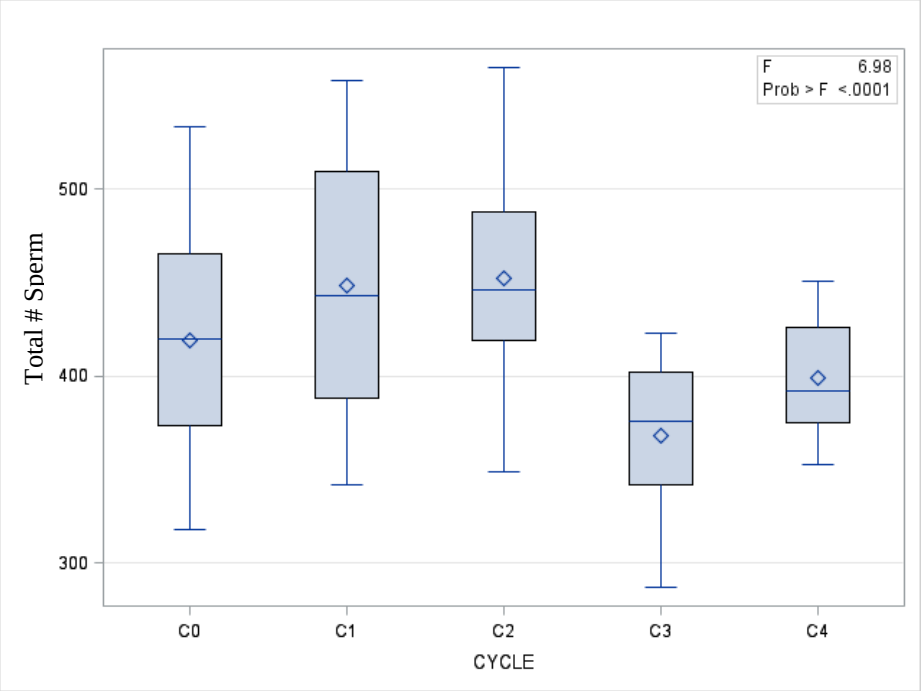

Figure 4.3 The number of sperm counted in the medial lobe in each gonotrophic cycle in *Ae. aegypti*. C0 = The number of sperm present before egg laying. C1-C4 sperm counts after egg laying cycle. The diamonds represent the mean amount of sperm, the median is represented by the blue horizontal lies and the extended lines from the boxes are the maximum and minimum values of

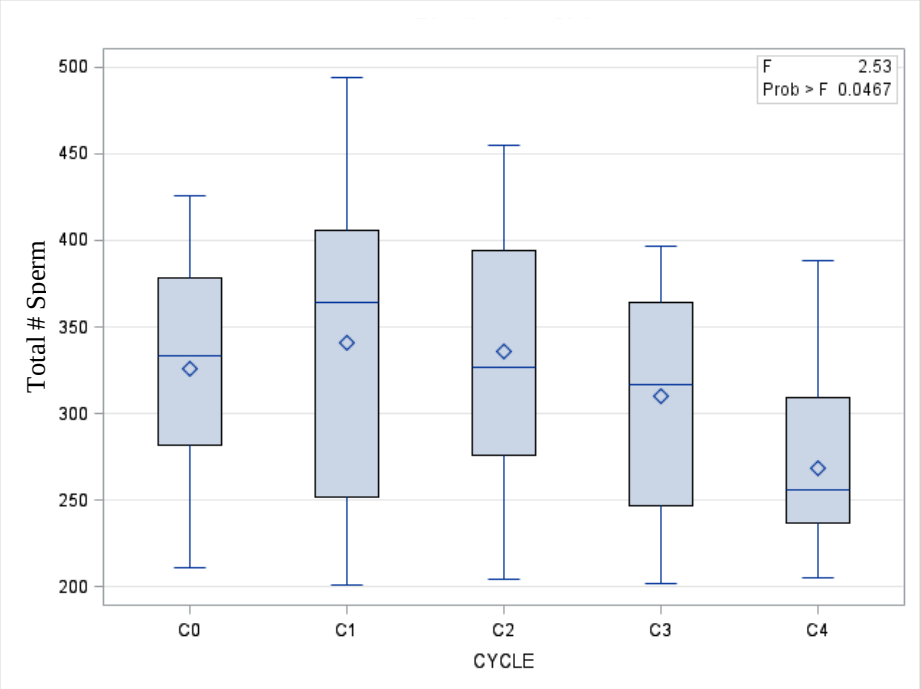

Figure 4.4 The number of sperm counted in the lateral lobe in each gonotrophic cycle in *Ae. aegypti*. C0 = The number of sperm present before egg laying. C1-C4 sperm counts after egg laying cycle. The diamonds represent the mean amount of sperm, the median is represented by the blue horizontal lies and the extended lines from the boxes are the maximum and minimum values of sperm observed

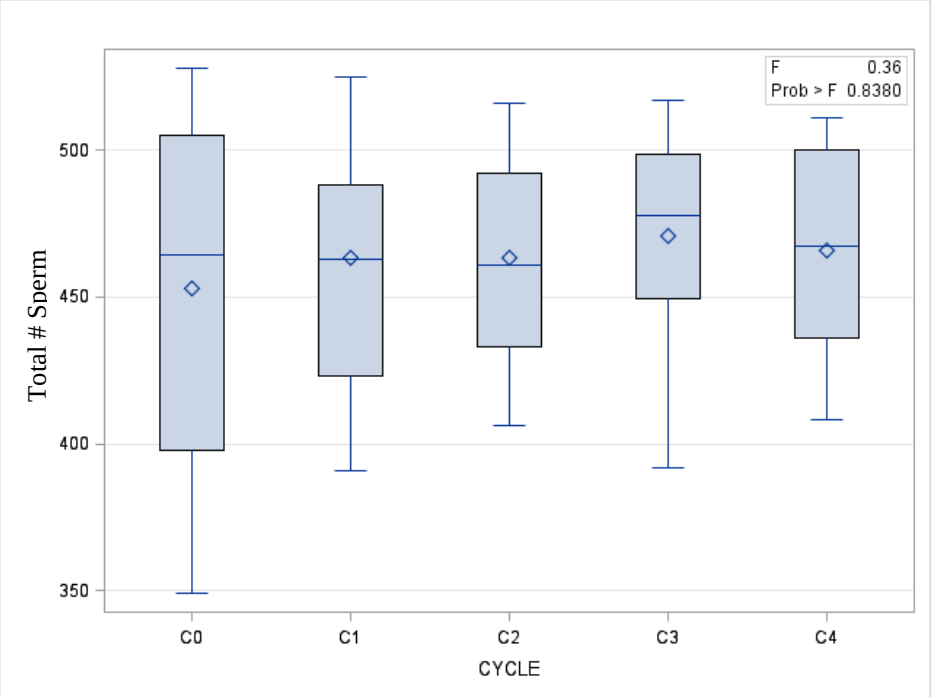

Figure 4.5 The number of sperm counted in the medial lobe in each gonotrophic cycle in *Ae. albopictus*. C0 = The number of sperm present before egg laying. C1-C4 sperm counts after egg laying cycle. The diamonds represent the mean amount of sperm, the median is represented by the blue horizontal lies and the extended lines from the boxes are the maximum and minimum values of sperm observed in each cycle.

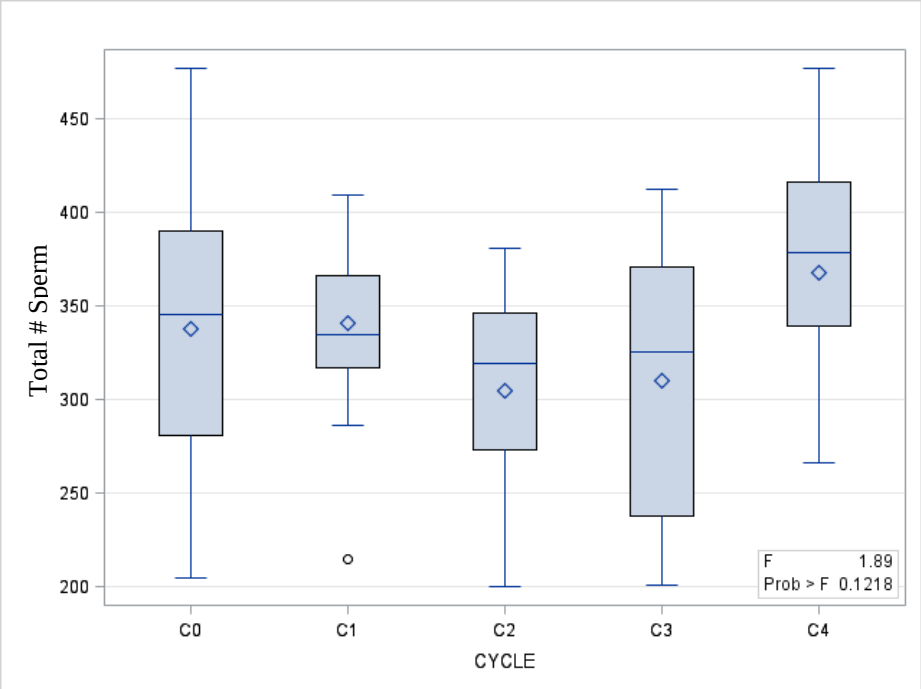

Figure 4.6 The number of sperm counted in the lateral lobe in each gonotrophic cycle in *Ae. albopictus*. C0 = The number of sperm present before egg laying. C1-C4 sperm counts after egg laying cycle. The diamonds represent the mean amount of sperm, the median is represented by the blue horizontal lies and the extended lines from the boxes are the maximum and minimum values of sperm observed in each cycle.

References

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., and Wolfner, M.F. 2011. Insect seminal fluid proteins: identification and function. Annual review of entomology, 56, 21.

Boyer, S., Toty, C., Jacquet, M., Lempérière, G., Fontenille, D. 2012. Evidence of multiple inseminations in the field in *Aedes albopictus*. PloS One 7(8):e42040.

Clements, A.N. 1999. The biology of mosquitoes. Wallingford, Oxon, UK ; Cambridge, MA: CABI.

Eberhard, W.G. 1996. Female control: Sexual selection by cryptic female choice. Princeton, N.J: Princeton University Press.

Farjana, T., and Tuno, N. 2013. Multiple blood feeding and host-seeking behavior in *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae). Journal of medical entomology, 50(4), 838-846.

Fritz, A.H., and Turner F.R. 2002. A light and electron microscopical study of the spermathecae and ventral receptacle of Anastrepha suspensa (Diptera: Tephritidae) and implications in female influence of sperm storage. Arthropod Structure & Development, 30(4), 293-313.

Gillott, C. 2003. Male accessory gland secretions: modulators of female reproductive physiology and behavior. Annual Review of Entomology, 48(1), 163-184.

Gwadz, R.W., and Craig Jr, GE. 1970. Female polygamy due to inadequate semen transfer in *Aedes Aegypti* 1'2. Mosquito News, 30(3).

Helinski, M.E., Valerio, L., Facchinelli, L., Scott, T.W., Ramsey, J., Harrington, L.C. 2012. Evidence of polyandry for *Aedes aegypti* in semifield enclosures. Am J Trop Med Hyg 86(4):635-641.

Helinski, M.E., and Harrington L.C. 2011. Male mating history and body size influence female fecundity and longevity of the dengue vector Aedes aegypti. Journal of medical entomology, 48(2), 202-211.

Jones, J.C., and Wheeler, R.E. 1965a. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. description. Biol Bull 129(1):134-150.

Klowden, MJ., and Chambers, G.M. 1992. Reproductive and metabolic differences between *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). J. Med. Entomol, 29(467), 47.

Linley, J., and Simmons, K. 1981. Sperm motility and spermathecal filling in lower Diptera. International Journal of Invertebrate Reproduction 4(2):137-145.

Oliva, C.F., Damiens, D., Vreysen, M.J., Lemperière, G., and Gilles, J. 2013. Reproductive strategies of *Aedes albopictus* (Diptera: Culicidae) and implications for the sterile insect technique.

Pascini, TV., Ramalho-Ortigão, M., Martins, G.F. 2012. Morphological and morphometrical assessment of spermathecae of *Aedes aegypti* females. Memórias do Instituto Oswaldo Cruz 107(6):705-712.

Perez-Staples, D., Harmer, A.M.T., Taylor, P.W. 2007. Sperm storage and utilization in female queensland fruit flies (*Bactrocera tryoni*). Physiol Entomol 32(2):127-35.

Pitnick, S., and García–González, F. 2002. Harm to females increases with male body size in *Drosophila melanogaster*. Proceedings of the Royal Society of London B: Biological Sciences, 269(1502), 1821-1828.

Ponlawat, A., and Harrington, L.C. 2009. Factors associated with male mating success of the dengue vector mosquito, *Aedes aegypti*. Am J Trop Med Hyg 80(3):395-400.

Ram, K.R., and Wolfner, M.F. 2007. Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. Integrative and Comparative Biology, 47(3), 427-445.

Renshaw, M., Service, M., and Birley, M. 1994. Size variation and reproductive success in the mosquito *Aedes cantans*. Med Vet Entomol 8(2):179-186.

Roth, L.M. 1948. A study of mosquito behavior - an experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). Am Midl Nat 40(2):265-352.

Sirot, L.K., Poulson, R.L., McKenna, M.C., Girnary, H., Wolfner, M.F., Harrington L.C. 2008. Identity and transfer of male reproductive gland proteins of the dengue vector mosquito, *Aedes aegypti*: potential tools.

Ward, P.I. 2000. Cryptic female choice in the yellow dung fly *Scathophaga stercoraria* (L.). Evolution 54(5):1680-1686.

Ward, P.I. 1993. Females influence sperm storage and use in the yellow dung fly *Scathophaga stercoraria* (L.). Behav Ecol Sociobiol 32(5).

Wiklund, C., and Kaitala, A. 1995. Sexual selection for large male size in a polyandrous butterfly: the effect of body size on male versus female reproductive success in *Pieris napi*. Behavioral Ecology, 6(1), 6-13.

Yuval, B., Blay, S., and Kaspi, R. 1996. Sperm transfer and storage in the Mediterranean fruit fly (Diptera: Tephritidae). Annals of the Entomological Society of America, 89(3), 486-492.

Conclusions

In this thesis I discussed the potential uses of multiple lobed spermathecae in *Ae. aegypti* and *Ae. albopictus*. I concluded that body size, heterospecific mating and lobe usage preference were not factors that influenced lobe filling or selection. However the reproductive physiology is still poorly understood in these species. Understanding the reproductive physiology will provide knowledge for future genetic and molecular sterilization techniques to be developed. Further investigations focused on the female reproductive tract and seminal protein fluids (Spfs) need to be conducted.

Future studies should examine the bursa inseminalis. When males of *Ae. aegypti* and *Ae. albopictus* inseminate females, sperm and Spfs are deposited into the bursa inseminalis. Afterwards sperm are transferred into the spermatheca (Jones and Wheeler 1965). Only about two thirds of sperm deposited are taken into the spermatheca (Jones and Wheeler 1965). The remaining sperm and Spfs in the bursa inseminalis form a dense granular mass (Oliva et al 2013, Spielman et al 1969). The mass remains in the bursa inseminalis for 40min to 6hrs (Oliva et al 2013). Afterwards the mass dissolves within 24-48hrs and the bursa inseminalis is empty. One hypothesis proposed is that the dense mass acts as an initial barrier to further insemination (Oliva et al 2013). The mass formed is thought to be similar to *Anophele gambiae* which seminal protein fluids form a mating plug (Avila et al 2011).

Seminal protein fluids of *Ae. aegypti* and *Ae. albopictus* need to be studied further. Seminal protein fluids are responsible for variety of physiological and behavioral changes in both species. Since Spfs are transferred to female during copulation they interact with the bursa inseminalis, the interactions between them is unknown. Seminal protein fluids in *Ae.*

aegypti and *Ae. albopictus* prevent females from remating, increase fecundity and promote sperm viability (Avila et al 2011, Helinski et al 2012). In heterospecific matings, Spfs from *Ae. albopictus* males prevented *Ae. aegypti* females from remating but not in the reverse cross (Tripet et al 2011). Identifying the function of individual Spfs would provide insight on how they influence female behavior and interact with the female reproductive tract. Recent bioinformatics studies have started to identify what functions these proteins might be and require further examination (Boes et al 2014).

Overall future studies on the bursa inseminalis and seminal protein fluids should be conducted. The bursa inseminalis and Spfs directly interact with one another. Current research has begun identifying potential functions of Spfs but have yet to be confirmed in *Ae. aegypti* and *Ae. albopictus*. Investigations focused on these topics may lead to new sterilization techniques.

References

Boes, K. E., Ribeiro, J. M., Wong, A., Harrington, L. C., Wolfner, M. F., and Sirot, L. K. (2014). Identification and characterization of seminal fluid proteins in the asian tiger mosquito, aedes albopictus

Avila, F.W., Sirot, L.K., LaFlamme, B.A., Rubinstein, C.D., and Wolfner, M.F. 2011. Insect seminal fluid proteins: identification and function. Annual review of entomology, 56, 21.

Helinski, M.E., Deewatthanawong, P., Sirot, L.K., Wolfner, M.F., Harrington, L.C. 2012. Duration and dose-dependency of female sexual receptivity responses to seminal fluid proteins in *Aedes albopictus* and *Ae. aegypti* mosquitoes. J Insect Physiol 58(10):1307-1313.

Jones, J.C., and Wheeler, R.E. 1965. Studies on spermathecal filling in *Aedes aegypti* (Linnaeus). I. description. Biol Bull 129(1):134-150.

Oliva, C.F., Damiens, D., Vreysen, M.J., Lemperière, G., and Gilles, J. 2013. Reproductive strategies of *Aedes albopictus* (Diptera: Culicidae) and implications for the sterile insect technique.

Spielman, A., Leahy, M.G., and Skaff, V. 1969. Failure of effective insemination of young female Aedes aegypti mosquitoes. Journal of insect physiology, 15(9), 1471-1479.

Tripet, F., Lounibos, L.P., Robbins, D., Moran, J., Nishimura, N., Blosser, E.M. 2011. Competitive reduction by satyrization? Evidence for interspecific mating in nature and asymmetric reproductive competition between invasive mosquito vectors. Am J Trop Med Hyg 85(2):265-270.