REPRODUCTIVE INTERFERENCE AND SPERMATHECAL USE IN INVASIVE MOSQUITO VECTORS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2017
To the loving memory of my parents, Tita and Tito
ACKNOWLEDGMENTS

I’m very grateful to my advisor, Phil Lounibos, and my co-advisor, Jorge Rey, for giving me the opportunity to come and study at the Florida Medical Entomology Laboratory, for their continued guidance, support, unwavering encouragement and valuable insight. Phil introduced me to the topic of reproductive interference between invasive mosquitoes, and guided me to develop interesting research on this area. I want to express my gratitude to my committee members, Dr. Dan Hahn and Dr. Jane Brockmann, for their insightful feedback in the development of my studies.

I want to thank the professors from the Florida Medical Entomology Laboratory. Chelsea Smart who guided me with molecular biology trials, and let me work in her lab, and professors Cynthia Lord, Barry Alto, Nathan Burkett-Cadena, Dagne Duguma, George O’Meara and Roxanne Connelly for helpful discussions.

I acknowledge the valuable advice and assistance of Dr. Jason Curtis, from the Light Stable Isotope Mass Spectrometry Laboratory in the Department of Geological Sciences at the University of Florida, who ran the isotope samples. I want to thank James Colee, from IFAS Statistics Consulting Services, for his help with statistics in the experiment about spermathecal use.

I was fortunate to be at a wonderful lab. I want to express my gratitude to Nildimar Honorio who invited me to participate in her research on reproductive interference with Brazilian mosquitoes and gave me professional and personal advice. I want to thank Naoya Nishimura for his support in diverse aspects of my research and his help with statistics. I’m grateful for having the opportunity to participate in interesting projects with Irka Bargielowski. I’m thankful to Tom Swan for his collaboration with my experiments and his constant motivation, to Sarah Murr for her help with the
development of my experiments, and to Joy Anogwih for her encouragement in the last part of my PhD.

I want to thank each one of the members of the Florida Medical Entomology Laboratory. I want to express my gratitude to Tanise Stenn, John Crosby, Sheila O’Connell, Sara Ortiz, Carol Thomas, Carolina Acevedo and Karen Garrett-Krauss, who were a great help in different moments. I’m thankful to former and current students, Eva Buckner, Erik Blosser, Kylie Zirbel, Isaiah Hoyer, Kristin Sloyer, Shawna Bellamy, Rebecca Zimler, Bethany McGregor, Casey Parker, Gaby Blohm and Luana Farnesi, because it was very important for my development to have the opportunity to be around other students at the lab. I want to thank my friends, Camila Pizano, Elena Ortiz and Juan Pablo Gómez for their continued motivation. I want to express my gratitude to my family for their unconditional support.

I want to acknowledge the National Institutes of Health, Colciencias, and the Entomological Society of America-Monsanto Research grant award for the financial support of my studies and research.
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The mosquitoes *Aedes aegypti* and *Aedes albopictus* are highly invasive and the primary vectors to humans of dengue, chikungunya, and Zika viruses. These two ecologically similar species have evolved independently in their native ranges, *Ae. aegypti* in Africa and *Ae. albopictus* in Asia. Where their ranges overlap, interspecific mating is facilitated by both species swarming to mate around bloodmeal hosts during daylight. However, the species differ in their reproductive biology.

In southeastern USA and Bermuda, abrupt declines in range and abundance of *Ae. aegypti* were associated with invasions and spread of *Ae. albopictus*. Satyrization, a form of reproductive interference in which interspecific matings occur, has been suggested as a possible mechanism to explain such competitive displacements of *Ae. aegypti*. Low frequencies of interspecific mating between these two species have been detected in wild-caught females on four continents. It is also known that heterospecific male accessory gland substances transferred during mating trigger refractoriness to further mating in *Ae. aegypti*, but not in *Ae. albopictus* females.
I developed a technique to detect insemination status in live *Ae. aegypti* females. The application of this technique demonstrated that some *Ae. aegypti* females, previously exposed to *Ae. albopictus* males, were rendered refractory to subsequent conspecific mating even if their spermathecae contained no heterospecific sperm. I also studied spermathecal usage in *Ae. aegypti* and *Ae. albopictus*. My results showed that significantly more spermathecae contained sperm in large than in small *Ae. albopictus* females, but there was no effect of *Ae. aegypti* female body size on the average number of spermathecae used.

In conclusion, a new technique was developed to detect insemination status in live *Ae. aegypti* females. This technique helped to show that satyrization could occur without successful insemination of *Ae. aegypti* by *Ae. albopictus*. Interspecific differences in the effect of female size on the number of spermathecae containing sperm imply differences in the mating biology of these two species. My work strengthens the understanding of reproductive interference as a mechanism for competitive displacements of *Ae. aegypti* by *Ae. albopictus* and provides important new information on the mating biology of these two species.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Origin and Spread of *Ae. aegypti* and *Ae. albopictus*

*Aedes aegypti* and *Ae. albopictus* belong to the subgenus *Stegomyia*. *Aedes aegypti* originated in Africa, where two subspecies are recognized, *Ae. aegypti aegypti* and *Ae. aegypti formosus* (Christophers, 1960; Tabachnick, 1991). The domestic form, *Aedes aegypti aegypti*, arose in Africa from the sylvan ancestor, *Aedes aegypti formosus* (Brown et al., 2014). It has been hypothesized that the subspecies *Ae. aegypti aegypti* originated in North Africa from an isolated population of *Ae. aegypti formosus* during a drought period. The dry conditions may have selected for mosquitoes with domesticated behaviors, such as the use of human containers as larval habitats (Tabachnick, 1991). However, the exact origin of *Ae. aegypti aegypti* in Africa is unresolved (Brown et al., 2014). In this document, *Ae. aegypti* will be used as a synonym for *Ae. aegypti aegypti*.

*Aedes aegypti*, known as the yellow fever mosquito, was most likely introduced to the New World during the XV through XVII centuries, transported from Africa on ships that were used as part of the slave trade. However, it may have also arrived to the New World on ships coming from Spain and Portugal, where it may have been first introduced from Africa (Tabachnick, 1991). Its spread into Asia occurred in the second half of the nineteenth century (Smith, 1956; Tabachnick, 1991), and to Australia in the late XVIII century or in the mid-XIX century (Mackenzie et al., 1996). Molecular analyses support the belief that *Ae. aegypti* moved first from Africa into the New World, followed by a secondary invasion from the New World into the Southeast Asia and the Pacific (Brown et al., 2014).
In the early 1900s *Ae. aegypti* was found in all the countries of the American continent, except Canada (OPS, 1995). The *Ae. aegypti* eradication program implemented by the Pan American Health Organization (PAHO) in the mid-1900s eliminated *Ae. aegypti* in 18 countries in the Americas. However, in some countries including, the United States, Venezuela, and some Caribbean Islands it was never eradicated (OPS, 1995). During the 1970s and 1980s, *Ae. aegypti* reestablished populations in the countries where it was eliminated previously (Gubler, 1998).

*Aedes albopictus*, commonly known as the Asian tiger mosquito, is native to Southeast Asia and has spread to many regions of the world, particularly since the 1980s. It is believed that centuries ago, *Ae. albopictus* spread to the smaller Indian Ocean Islands and to Madagascar (Enserink, 2008). In the late nineteenth century *Ae. albopictus* arrived to the Pacific islands (Hawley, 1988; Lounibos, 2002). However, its largest spread started in the 1980s and is associated with modern shipping and the trade of rubber tires. In the continental United States, *Ae. albopictus* became established in Houston, Texas in the mid 1980s (Sprenger and Wuithiranyagool, 1986). Then it spread rapidly throughout the eastern states (Hawley, 1988; Moore, 1999). It has also been found in western states, like California, were it remains established in LA County (Metzger and Hu, 2012; Zhong et al., 2013).

*Aedes albopictus* has also invaded the Caribbean, Central and South America, Europe, and Africa (Enserink, 2008; Kraemer et al., 2015). In Latin America, the species was found in Brazil in 1986 (Forattini, 1986) and it spread throughout the country (Santos, 2003). Following its establishment in the United States and Brazil, *Ae. albopictus* invaded many Latin American countries from Mexico to Argentina, including
the Caribbean islands (Benedict et al., 2007). In Europe, it was first discovered established in Albania in 1979 (Adhami and Reiter, 1998) and it has invaded several countries (Benedict et al., 2007; Enserink, 1998). In Africa, it was introduced in Nigeria in the 1990s (Savage et al., 1992), and is currently established in Cameroon (Fontenille and Toto, 2001), Equatorial Guinea (Toto et al., 2003), Gabon (Enserink, 2008), Central African Republic (Diallo et al., 2010), and Republic of Congo (Kelvin, 2011).

**Public Health Importance of *Ae. aegypti* and *Ae. albopictus***

*Aedes aegypti* and *Ae. albopictus* are vectors of important arboviruses, including dengue, chikungunya and Zika. *Aedes aegypti* is the primary vector of dengue in most of the world. *Aedes albopictus* is currently the primary dengue vector in rural areas of India and Southeast Asia (Foster and Walker, 2009), in urban southern China (Li et al., 2014), in Gabon (Paupy et al., 2010), and in Hawaii (Effler et al., 2005). The disease manifests either as classic dengue fever or more severe dengue hemorrhagic fever and dengue shock syndrome (Foster and Walker, 2009). It is estimated that around 390 million dengue infections occur per year, of which 96 million manifest clinically (Bhatt et al., 2013), including around 500,000 cases with severe dengue that require hospitalization. About 2.5% of people affected by severe dengue die (WHO, 2016a).

*Aedes aegypti* is also the most important vector of chikungunya virus (CHIKV) in some urban areas of Asia and the East African mainland (Foster and Walker, 2009), and recently in the Americas (Morrison, 2014). *Aedes albopictus* is recognized as the primary vector of CHIKV in the Indian Ocean Islands, Italy (Foster and Walker, 2009), Gabon (Paupy et al., 2010), Cameroon (de Lamballerie et al., 2008), and the Indian state of Kerala in 2007 (Morrison, 2014). *Aedes aegypti* was first recognized as the traditional vector of epidemic CHIKV. Transmission of epidemic CHIKV by *Ae.*
*albopictus* was facilitated by a single mutation in the viral envelope protein (Tsetsarkin et al., 2007).

The word chikungunya derives from the Kimakonde language and means “to become contorted”. Chikungunya is characterized by fever and joint pain. Other common symptoms include muscle pain, headache, nausea, fatigue and rash (WHO, 2016b). Lethal cases may occur. In epidemics occurring in the Indian Ocean region in 2005-2006 and in India in 2006-2007, unusual clinical complications and fatal cases were reported (Renault et al., 2008; Sourisseau et al., 2007; Zeller et al., 2016).

Zika virus was little known until 2007, when it caused an outbreak in the Pacific Island of Yap (Duffy et al., 2009). Previously, circulation of Zika virus was limited to tropical Africa and parts of Southeast Asia, where no outbreaks and only few known human cases of Zika virus infections and disease manifestation had been documented (Fagbami, 1979; Duffy et al., 2009; Olson et al., 1981; Simpson, 1964;). In the last decade, outbreaks have been reported in several Islands in Oceania (Duffy et al., 2009; ECDC, 2015), and the Americas (WHO, 2017a). Vectors of Zika virus include the African sylvan vectors *Ae. africanus* and *Ae. luteocephalus* (Diallo et al., 2014; Haddow et al., 1964; McCrae and Kirya, 1982; Weinbren and Williams, 1958), the African vectors *Ae. furcifer* and *Ae. vittatus* that circulate between the forest and villages (Diallo et al., 2014), and the non-sylvan vectors *Ae. aegypti* and *Ae. albopictus* (Chouin-Carneiro et al., 2016; Marchette et al., 1969; Weger-Lucarelli et al., 2016). Outside of Africa, the most probable vectors of Zika virus are *Ae. aegypti* (Marchette et al., 1969), and *Ae. albopictus* (Lounibos et al., 2016a).
People with Zika usually have symptoms of rash, mild fever, joint pain, headache and conjunctivitis (Duffy et al., 2009; Simpson, 1964). In recent epidemics, Zika virus also has been associated with microcephaly in babies born to Zika-infected mothers, and with Guillain-Barré Syndrome (CDC, 2017a; PAHO, 2017), in which the person’s immune system attacks their nerve cells, causing limb weakness and paralysis (Millon, 2015; Yuki, 2001).

*Aedes aegypti* is also the classical vector of urban yellow fever (YF). Throughout the 18th and 19th centuries, YF was a devastating disease with periodic outbreaks in the Americas, Europe (Rogers et al., 2006), and Africa (Boyce, 1911). The production of an effective yellow fever vaccine in the 1930s, diminished urban yellow fever transmission. In the Americas the *Ae. aegypti* eradication program in the mid-1900s reduced vector populations in most of its range. However, the disease persisted, mainly in forest areas, where sylvatic vectors, like *Haemagogus* species in the Americas and *Ae. africanus* and *Aedes (Diceromyia)* spp. in Africa are involved (Monath, 1986; Rogers et al., 2006). Human cases since 1950s have been mainly reported in persons associated with the forest. However, since the mid 1980s epidemic yellow fever has reemerged in West Africa (Gubler, 1998), and outbreaks have been reported in East Africa (Onyango et al., 2004; Sanders and Tukei, 1996). In the Americas, the last urban epidemic was reported in 1942 (Monath, 1986), however urban epidemics may occur as *Ae. aegypti* has reinfested many urban centers of the American tropics (Gubler, 1998). Since December 2016 ongoing outbreaks of yellow fever have been occurring in Brazil, mainly in rural areas (CDC, 2017b; WHO, 2017b). People with yellow fever have high fever, headache, nausea, and muscle pain. Around 15% of people infected with yellow
fever, develop a more severe form of the disease, characterized by necrosis in the liver, jaundice, and hemorrhage. Mortality in patients that enter the severe phase is around 20–50% (CDC, 2015; Foster and Walker, 2009).

**Characteristics of Ae. aegypti and Ae. albopictus**

*Aedes aegypti* and *Ae. albopictus* have evolved independently (Leahy and Craig, 1967). They belong to different groups of the subgenus *Stegomyia*. *Aedes aegypti* is part of the *Aegypti* Group originally from the Afrotropical Region (Huang, 2004), and *Ae. albopictus* belongs to the *Scutellaris* Group, native to Southeast Asia and Pacific Islands (Huang, 1972). They are ecologically similar species (Murrell et al., 2011) that lay their eggs in artificial and natural containers, such as discarded tires, water pots, barrels, and tree holes (Clements, 1999; Hawley, 1988). In sympatry both species can share the same larval habitats, and therefore co-occurrence of larvae and pupae in the same container may be common, especially in suburban areas (Braks et al., 2003). *Aedes aegypti* is more associated with urbanized locations that have pavement and buildings, and less with vegetated sites. *Aedes albopictus* is more common in rural settings that have ground or canopy vegetation and unpaved roads (Rey et al., 2006). Both species share two important adaptations that make them especially suited to human transport and to the invasion of new habitats: a tendency to oviposit in human-provided containers, and the ability of their eggs to remain viable and hatch after long periods of desiccation (Winchester and Kapan, 2013).

Adults of both species aggregate or lek near vertebrate hosts. Females seek hosts in order to get a blood meal, and males to attempt to copulate (Clements, 1999). In both species peaks of flight activity, swarm formation, and mating occur shortly after dawn (Gubler and Bhattacharya, 1972; Hartberg, 1971) and before dusk (Basio and
Magluyan, 1975; Clements, 1999). Females are generally considered monandrous, as they only mate once. Refractoriness to further mating is triggered by male accessory gland proteins that are transferred to females during mating (Clements, 1999; Craig, 1967; Klowden, 1999). Sperm transferred by one male are stored permanently in the spermathecae and are sufficient to fertilize eggs for the rest of a female's life (Jones, 1968; Oliva et al., 2013). Differences in the mating biology of *Ae. aegypti* and *Ae. albopictus* have been detected in relation to fecundity and sperm storage. *Aedes albopictus* store more sperm in spermathecae than *Ae. aegypti* females, and fecundity increases occur in *Ae. albopictus* females, that mate with larger males, but not in *Ae. aegypti* (De Jesus and Reiskind, 2016).

**Spread of *Ae. albopictus* and Reduction of *Ae. aegypti***

In some regions the decline of *Ae. aegypti* populations after the arrival of *Ae. albopictus* has been documented (Kaplan et al., 2010; Hobbs et al., 1991; Lounibos, 2002; Mekuria and Hyatt, 1995; Nasci et al., 1989; O'Meara et al., 1993; 1995). This phenomenon has been reported in Bermuda (Kaplan et al., 2010), Honolulu, Hawaii (Winchester and Kapan, 2013), and the southeastern USA, including Florida (O'Meara, et al., 1993, 1995), Louisiana (Nasci et al., 1989), Alabama (Hobbs et al., 1991), and South Carolina (Mekuria and Hyatt, 1995).

In Florida, *Ae. aegypti* used to have a broad distribution, but became rare or disappeared after the arrival of *Ae. albopictus*, or became restricted to urban areas of the South (O'Meara et al., 1995). *Aedes albopictus* was first found in the state in 1986 (Peacock et al., 1988) and by 1994 it had spread into all 67 counties. At several scrap tire sites and in other sites with abundant artificial water-holding containers, major declines in *Ae. aegypti* populations occurred after the invasion of *Ae. albopictus* in both
rural and urban areas of northern and central Florida and in rural areas of the south part of the state (O’Meara et al., 1995). The spread of *Ae. albopictus* in scrap tires and other artificial water-holding containers was monitored in ten locations along Route 441 in Florida from 1991 to 1994 by O’Meara et al. (1995). In 1991, *Ae. albopictus* was the only species collected at the three northernmost cities (Lake City, Gainesville, Ocala), and *Ae. aegypti* was the only species found at the three southernmost locations (St. Cloud, Yeehaw Junction, Okeechobee). By 1994, *Ae. albopictus* was dominant at all ten locations, and only two locations (Apopka and Okeechobee) harbored some *Ae. aegypti* (Figure 1-1).

In Hawaii and Bermuda the relative abundance of both species was also monitored (Kaplan et al., 2010; Winchester and Kapan, 2013). In Hawaii, *Ae. aegypti* was introduced around 1892. *Aedes albopictus* was found 5-15 years later. Surveys done in Honolulu, during the years 1910-1920, showed a trend of *Ae. albopictus* displacing *Ae. aegypti*. In 1943, *Ae. aegypti* presented a small recovery in Honolulu, however since 1949 it was not found anymore (Winchester and Kapan, 2013). In Bermuda, *Ae. albopictus* was first detected in 2000, and thereafter a steep decrease in *Ae. aegypti* abundance was observed during the years 2000-2002 (Kaplan et al., 2010).

**Explanations for the Decline of *Ae. aegypti***

Several mechanisms have been suggested to explain competitive displacements of *Ae. aegypti* by *Ae. albopictus* in the United States and Bermuda, including apparent competition (Craig, 1993), hatching inhibition of *Ae. aegypti* eggs caused by *Ae. albopictus* larvae (Edgerly et al., 1993), larval competition (Juliano, 1998) and reproductive interference (Nasci et al., 1989).
Apparent competition is defined as an indirect interaction, in which shared natural enemies (parasites, predators) have differential effects on two species (Holt and Lawton, 1994). *Aedes albopictus* and *Ae. aegypti* are parasitized by different protozoan species of the genus *Ascogregarina*. *Aedes albopictus* is parasitized by *A. taiwanensis* and *Ae. aegypti* by *A. culicis* (Craig, 1993). Each species can tolerate its associated *Ascogregarina* species, but if infestations are heavy, mortality of 10 and 25% can occur (Craig, 1993). Cross-species *Ascogregarina* infections (*Aedes albopictus* parasitized by *A. culicis* and *Ae. aegypti* parasitized by *A. taiwanensis*) have different effects on each mosquito species. *Ascogregarina taiwanensis* can produce harmful effects on *Ae. aegypti* (Munstermann and Wesson, 1990), but mortality is not so intense in the reciprocal cross-infection (Craig, 1993). However, field experiments done by Juliano (1998) showed that the frequency of larvae parasitized with protozoans from the genus *Ascogregarina* was significantly greater for *Ae. albopictus* (21.8%: 31/142) than for *Ae. aegypti* (0.8%: 1/117) larvae. Thus, apparent competition does not support the decline of *Ae. aegypti*.

A second mechanism, egg hatching inhibition, was studied in the laboratory by Edgerly et al. (1993). *Aedes albopictus* has a greater ability than *Ae. aegypti* to suppress egg hatching of congeneric eggs. Furthermore, *Ae. albopictus* eggs present the lowest inhibition when they are exposed to high larval densities, regardless of the species (*Ae. albopictus*, *Ae. aegypti*, *Ae. triseriatus*). Based on this laboratory experiment, Edgerly et al. (1993) suggested that larva-induced hatch inhibition has the potential to influence the decline of *Ae. aegypti* following invasion by *Ae. albopictus*. 
Another mechanism to explain the decline of *Ae. aegypti* has been larval competition. The outcome of interspecific competition between *Ae. aegypti* and *Ae. albopictus* is associated with food quality, particularly its nitrogen content and rate of detritus decay (Juliano, 2009; 2010). High quality food (e.g. dead insects, liver powder), has greater total nitrogen, and higher decay rates than low quality food (leaf litter) (Yee and Juliano, 2006). *Aedes albopictus* has a competitive advantage, under low food quality conditions (more typical of field larval habitats). High quality resources tend to give competitive equality to both species or advantage for *Ae. aegypti* (Juliano 2009; 2010; Lounibos, 2007). Among the possible mechanisms to explain the rapid displacement of *Ae. aegypti* by *Ae. albopictus*, interspecific larval competition was the most studied and accepted explanation in the 90s and in the early 2000s (Juliano, 1998; Juliano and Lounibos, 2005; Lounibos, 2002). However, the extremely rapid disappearance of *Ae. aegypti* after the arrival of *Ae. albopictus* could not be explained by larval competition alone (Kaplan et al., 2010).

Reproductive interference, specifically satyrization, is a fourth mechanism that was proposed to understand the decline of *Ae. aegypti* (Nasci et al. 1989). Reproductive interference is an interspecific interaction that occurs when organisms do not discriminate heterospecies from their own and it adversely affects the fitness of at least one of the species involved. Satyrization is a type of reproductive interference, in which interspecific mating, yielding no progeny, occurs (Gröning and Hochkirch, 2008). Satyrization, was proposed as a mechanism to explain the displacement of *Ae. aegypti* by *Ae. albopictus*, in the late 80s (Nasci et al., 1989), and has been studied
experimentally since 2010 (Bargielowski et al., 2013; 2015a; 2015b; Bargielowski and Lounibos, 2014; Lounibos et al., 2016b; Tripet et al., 2011).

Experiments showed that injections of heterologous male accessory gland substances triggered a switch to post-mating behavior in *Ae. aegypti* females, but not in *Ae. albopictus* females, in the same way that conspecific male accessory gland substances stimulate oviposition (Leahy and Craig, 1965) and refractoriness to mating (Tripet et al., 2011). Thus, fitness loss, after interspecific matings, is very high for *Ae. aegypti* females, as they are rendered refractory to further conspecific mating. In cage experiments, it has been documented that *Ae. aegypti* females from populations that are allopatric to *Ae. albopictus* are more susceptible to interspecific mating (insemination rates are higher) than *Ae. aegypti* females from sympatric populations, suggesting the evolution of resistance to satyrization, due to the high fitness loss for *Ae. aegypti* females of interspecific mating (Bargielowski et al., 2013). In laboratory studies it has also been shown that heterospecific inseminations are higher in crosses between *Ae. aegypti* females and *Ae. albopictus* males compared to inseminations between *Ae. albopictus* females and *Ae. aegypti* males, which is consistent with the unidirectional displacement that has occurred in the United States and Bermuda (Bargielowski et al. 2013; Nasci et al., 1989).

Interspecific matings between *Ae. aegypti* and *Ae. albopictus* yield no progeny. Leahy and Craig (1967) performed crosses in both directions, using several strains of *Ae. aegypti* and *Ae. albopictus* and no offspring were obtained from 156,466 eggs. Adults of *Ae. aegypti* and *Ae. albopictus* are likely to encounter each other in areas of sympathy. Similar frequencies of interspecific mating have been found in in wild-caught
females of both species on four continents (Bargielowski et al., 2015a; Tripet et al., 2011). In nature, cross-species matings between populations of *Ae. aegypti* and *Ae. albopictus*, measured as percentage of wild-caught females with heterospecific sperm in their spermathecae, have been low (1.12 - 3.73%). However, models predict that even low levels of reproductive interference, coupled with Lotka-Volterra competition, can lead to local extinctions and displacement (Kishi and Nakazawa, 2013; Ribeiro, 1988).

In my work, I focused on understanding satyrization as a mechanism to explain the rapid displacement of *Ae. aegypti* after the invasion of *Ae. albopictus* in the United States and Bermuda. In the first chapter, I describe a new technique that I developed, using microscopy, to detect the insemination status of live *Ae. aegypti* females. In the second chapter, I report on application of this technique to assess whether satyrization of *Ae. aegypti* females by *Ae. albopictus* males was occurring, even if heterospecific sperm were not transferred to *Ae. aegypti* spermathecae. In the third chapter, I examined the controls of spermathecal usage in these two species after intraspecific and interspecific matings.
Figure 1-1. Frequency of immature *Ae. aegypti* and *Ae. albopictus*. Collections were performed in scrap tires on a latitudinal transect on State Route 441 in Florida from 1991 to 1994 (Figure based on O’Meara et al., 1995).
CHAPTER 2
DETECTION OF INSEMINATION STATUS IN LIVE Aedes aegypti FEMALES

Introduction

Insemination status in female mosquitoes is typically assessed by dissections. Traditionally, the female is anesthetized and her genitalia with internal organs, including the spermathecae, are removed with forceps or needles under a dissecting microscope. Sperm, if present, are seen as a mass of threads in the spermathecae (Rosay, 1969). This traditional technique is very useful and reliable for checking presence or absence of sperm, e.g., to assess the timing of sexual receptivity after female emergence (Gwadz and Craig, 1968; Lounibos et al., 1996; O’Meara and Lounibos, 1981) or of mating after mark-release-recapture field experiments (Lounibos et al., 1998; Reisen and Aslamkhan, 1979). However, the procedure is fatal to the female, which cannot be kept alive for subsequent experiments or observations.

Females of most insects store and maintain sperm internally in the spermathecae (Gullan and Cranston, 2010; Simmons, 2001). Sperm in the spermathecae remain viable for prolonged periods of time, e.g., several years in the case of honey bees (Collins et al., 2004; Snodgrass, 1956) and ants (Tschinkel, 1987; Wheeler, 1960), and around one or two months in mosquitoes (Christophers, 1960). Most insect species have a single spermatheca (Gullan and Cranston, 2010; McAlpine et al., 1981), but the number of spermathecae varies from one to three among mosquito species (Yuval, 2006). Aedes aegypti females have three spermathecae (Christophers, 1960). Harbach

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and Knight (1980) prefer the term ‘spermathecal capsules’ for the reservoirs at the ends of the spermathecal ducts, which for simplicity herein I call spermathecae.

The new technique described here that allows detection of sperm in spermathecae of live insects, specifically in Ae. aegypti females, is facilitated by the distensibility of the female’s abdomen. Each segment of the abdomen of an adult mosquito is connected laterally by a pleural membrane, which continues unbroken throughout the length of the abdomen (Christophers, 1901; Harbach, 2014). The intersegmental membrane connects the tergum and sternum of adjacent abdominal segments (Harbach and Knight, 1980). The elasticity of these membranes allows the abdomen to become distended when the female takes a blood meal or when she is gravid and full of eggs (Christophers, 1960; Harbach, 2014).

**Methods**

**Source of Ae. aegypti.** Aedes aegypti were F5 progeny from collections in Key West, Florida. Mosquitoes were reared and maintained in an insectary at 27˚C, and 70% RH, and 14L:10D day length as described elsewhere (Bargielowski et al., 2013).

**Sperm check.** Each Ae. aegypti female was placed individually in a small cardboard container and lightly anesthetized with chloroform for approximately 10 seconds. Under a dissecting microscope, each female was placed ventral surface up on a glass slide, and the distal segments of the abdomen were placed in a drop of water to facilitate abdominal distention and to create a fluid medium for microscopic observations.

A square glass cover slip (15 x 15 mm), was lowered gently onto the abdomen, covering the VI- VIII segments and the genitalia (Figure 2-1). The pressure from the cover slip stretched the pleural and intersegmental membranes. After the cover slip was
positioned on the female’s abdomen, spermathecae were observed under a phase contrast compound microscope, using magnifications from 40 to 200x. In inseminated females, masses of motile spermatozoa were seen within the spermathecae (Figure 2-2). In uninseminated females, the spermathecae appeared empty (Figure 2-3). After checking for sperm, a few drops of water were applied with a pipette to the edges of the cover slip to facilitate its removal with fine jeweler’s forceps. Females were immediately returned to the individual containers and usually recovered from the procedure in 30-60 minutes. If the first sperm check was inconclusive, because abdominal distention was inadequate and/or spermathecae were not observed clearly, the procedure was repeated two or three times.

Validation of the technique. Two experiments were performed. In the first, the tester received in individual containers 60 females, whose histories were known only to a second party. The samples consisted of *Ae. aegypti* females that had been exposed for two weeks to conspecific males or two week old virgin females. Each female was checked for insemination, as described previously. In the second experiment, sperm detection was performed on *Ae. aegypti* females that had been exposed previously for three weeks to *Ae. albopictus* males. Under these conditions, approximately 50% of *Ae. aegypti* females from Key West are inseminated by *Ae. albopictus* (Bargielowski et al., 2013). After spermathecal observation on 97 live females, each was dissected by traditional, lethal techniques to compare results between the two procedures.

Survivorship. An experiment was performed to assess female survivorship after the procedure. To obtain specimens for a live sperm check, 150 *Ae. aegypti* females (Key West) and 150 *Ae. albopictus* males (F5, Vero Beach, FL), were exposed to one
another for three weeks in a cage. After spermathecal detections of live females, each was confined individually in a small cardboard container and observed every 24 hours for three days. A female was considered healthy if she was able to stand upright and fly. As controls, *Ae. aegypti* females exposed to the same conditions without spermathecal observations, were transferred directly to the cardboard containers and survivorship was assessed. All females had *ad libitum* access to a sugar water solution.

**Mating capacity.** An experiment was performed to determine mating capacity after the procedure. The tester received in individual containers four replicates of 20 females each, whose histories were known only to a second party. The replicates included *Ae. aegypti* that had been exposed for three weeks to conspecific males or three week old virgin females. Spermathecal observations on live females were performed. After females diagnosed to be virgin recovered from the procedure, each was exposed for 24 hours in an individual cage to two *Ae. aegypti* males. As controls, virgin *Ae. aegypti* females without spermathecal observations were exposed to two *Ae. aegypti* males under the same conditions. Four control replicates of 10 females were done. After 24 hours of exposure to males, traditional spermathecal dissections were done to confirm if females had been inseminated. All females had *ad libitum* access to a sugar water solution.

**Results and Discussion**

Experiments performed to validate the technique were completed successfully on the majority of the females. In the first experiment, the procedure was performed on 60 *Ae. aegypti* females, of which the presence or absence of sperm was resolved in 59, as 33 inseminated and 26 non-inseminated, for 100% accuracy. In the one unresolvable female, spermathecae were not observable because the terminal abdominal segments
were twisted and the abdomen did not distend normally. In the second experiment, the procedure was performed on 97 *Ae. aegypti* females exposed to *Ae. albopictus* males, of which the presence or absence of sperm was resolved in 94, for 98.9% accuracy (50 females positive for sperm in spermathecae and 43 females negative), as confirmed with the traditional dissecting technique. The assessment was incorrect (false negative) in one female (1.1%). For the three live females whose spermathecae were not observable, abdomens did not distend. The live observation of the only misdiagnosed female was probably inaccurate because she had relatively few sperm in only one of the small spermathecae. This mistake is not likely to occur in intraspecific matings, in which usually the large and one or two of the small spermathecae have sperm (Clements, 1999; Jones and Wheeler, 1965). In my observations I have noticed that in interspecific matings between *Ae. aegypti* females and *Ae. albopictus* males, females tend to store less sperm in the spermathecae, and patterns of spermathecal use differ from intraspecific matings (Tripet et al., 2011). I validated the technique and performed all the experiments with chloroform, however I anesthetized a few females using carbon dioxide. Under carbon dioxide anesthesia I obtained good abdominal distention and motile sperm were observable in spermathecae.

In the experiments performed to assess survivorship and mating capacity most females remained healthy after the procedure. In the experiment in which survivorship was tested, between 88 and 96% of the females subjected to the procedure survived with no apparent negative impacts for at least three days afterwards (Table 2-1). The rest of the females either died or were not able to stand upright and fly. Kaplan-Meier survival analysis showed no significant difference in survivorship among the three
classes: controls, inseminated and uninseminated females (Log Rank test: $X^2 = 3.68$ df =2, $P = 0.159$).

Although no significant differences were detected, the survivorship was a little lower in the uninseminated females, possibly because when spermathecae looked empty I repeated the procedure and the added manipulations may lead to a higher mortality. As I was applying the technique, I noticed that it is usually easier and faster to confirm presence of sperm in spermathecae rather than absence, as once sperm are observed the assessment is complete. If the spermathecae appear empty, it is recommended to repeat the procedure using a larger glass cover slip (18x18 mm to 25x25 mm), which facilitates distention of the abdomen and provides a better view. I use a smaller cover slip for initial observations because spermathecae with sperm may rupture easier than empty ones. When spermathecae are empty, breakage is unlikely. I also note that permanent access of caged females to sugar water facilitates improved abdominal distention.

Table 2-1. Percentage of healthy females after sperm check. Controls without sperm check.

<table>
<thead>
<tr>
<th>Female status (n)</th>
<th>Percentage of healthy females after sperm check</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>After 24 hours</td>
</tr>
<tr>
<td>Inseminated females (n=52)</td>
<td>96.1</td>
</tr>
<tr>
<td>Uninseminated females (n=56)</td>
<td>89.28</td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>100</td>
</tr>
</tbody>
</table>

In the experiment in which mating capacity was tested, most virgin females were capable of subsequent mating. After spermathecal observations were completed on live females, each uninseminated female (n=40) was exposed to two conspecific males. Approximately, 90 ($\pm 14.1$ s.d.) % of the females (n=36), survived and were able to stand upright and fly after their exposure to males. Of these, an average of 94.7 ($\pm 6.1$ s.d.)
s.d.) % mated successfully as evidenced by the presence of sperm in spermathecae. In the controls, 97.5 (±5 s.d.) % of the females were inseminated.

To date I have successfully applied this technique only on *Ae. aegypti* females. Its use on other mosquito species, or other Diptera and insects, will depend on spermathecal and sperm visibility through the distended abdomen, female body size and sclerotization, size of fat bodies and distensibility of the intersegmental and pleural membranes. This technique could be useful for studies that perform other procedures (after assessing insemination) on the body of the mosquito, e.g., experiments that examine the effect of mating status on body cuticle traits, such as cuticular hydrocarbons (Polerstock et al., 2002; Wagoner et al., 2014). In such laboratory studies, as it is necessary to keep the mosquito body for further procedures, it is usually assumed that females are mated if they have been exposed to males. With the new technique, if sperm presence is checked in live females, it will be possible to confirm insemination status, keeping the intact body. Also, it would be feasible to study the effect of mating status on chemical traits of field-collected mosquitoes. Detection of insemination status in live females could also be valuable in behavioral studies, e.g., experiments that assess the effect of insemination on locomotor activity (Jones and Gubbins, 1978; Lima-Camara et al., 2014). In such studies, insemination status is usually verified through dissections at the end of the experiment. This new technique would allow determination of insemination status in live females before performing behavioral tests.

This technique could have potential use in colonization and rearing of mosquito species in the laboratory, particularly those that are difficult to colonize or when few
adults are available to start a colony. For example, in species, such as *Mansonina annulata*, that have low adult emergence rates in the laboratory (Samung et al., 2006), it would be particularly useful to check if mating is taking place without losing adult females. Also, in experiments in which mated females are required for subsequent observations, e.g., oviposition and vertical transmission of arboviruses (Buckner et al., 2013), this new technique could be potentially useful. Most females that recovered after our procedures were capable of subsequent reproductive activities, but for purposes of colonization or arbovirus studies, it would be important to assess blood feeding and egg laying capacities, after the *in vivo* sperm check.

The few supplies and equipment needed are usually found in entomology laboratories. However, the technique may be considered as tedious, needing to be performed with care on each female, and sometimes it is necessary to relocate the cover slip several times on the mosquito’s abdomen until a good view of the spermathecae is obtained. Thus, it does not provide the quick assessment that is obtained with the conventional technique in which the spermathecae are dissected away from other tissues. My intention is not to compare this new technique with the traditional dissecting technique, as the two have different applications. This new technique allows confirmation of insemination status in live *Ae. aegypti* females, a procedure that had not been available previously.
Figure 2-1. Glass cover slip lowered onto the distal segments of the abdomen, covering the VI, VII and VIII abdominal segments and the genitalia. Dotted line marks the edge of the glass coverslip. Photo courtesy of María Cristina Carrasquilla Ferro.
Figure 2-2. Spermathecae containing sperm. The three spermathecae of *Ae. aegypti*, one large, and two smaller and lateral, are seen through the extended abdomen. In this photo, the two spermathecae that contain sperm are pointed by arrows. Photo courtesy of María Cristina Carrasquilla Ferro.
Figure 2-3. Spermathecae without sperm seen through the cover slip. Photo courtesy of María Cristina Carrasquilla Ferro.
CHAPTER 3
SATYRIZATION WITHOUT EVIDENCE OF SUCCESSFUL INSEMINATION FROM
INTERSPECIFIC MATING BETWEEN INVASIVE MOSQUITOES\(^1\)

Introduction

The mosquitoes *Aedes aegypti* and *Ae. albopictus* are the primary vectors
worldwide of dengue, chikungunya and Zika viruses (Chouin-Carneiro et al., 2016;
Foster and Walker, 2009; Marchette et al., 1969; Weger-Lucarelli et al., 2016). *Aedes*
aegypti is native to Africa but has spread cosmotropically primarily in the fifteenth to
seventeenth centuries (Lounibos, 2002). *Aedes albopictus* evolved in Asia and has
spread into expanded tropical and temperate ranges during the last three decades
(Benedict et al., 2007). Recent invasions by *Ae. albopictus* have been associated with
some rapid declines of *Ae. aegypti* such as in the southeastern USA and Bermuda
(Kaplan et al., 2010; Lounibos, 2002; Nasci et al., 1989).

Satyrization has been suggested as a possible mechanism for such declines
(Bargielowski et al. 2013; Nasci et al., 1989; Tripet et al., 2011). Satyrization is a form of
reproductive interference that reduces the fitness of at least one of the mating species
involved and is caused by incomplete species recognition (Gröning and Hochkirch,
2008; Ribeiro and Spielman, 1986). Adults of *Ae. aegypti* and *Ae. albopictus* encounter
each other in areas of sympatry, where low rates of cross-species matings (1.12 -
3.73\%) have been detected in nature, measured as percentages of females with
heterospecific sperm in their spermathecae (Bargielowski et al., 2015a; Tripet et al.,

\(^1\) Reprinted with permission from Carrasquilla, M.C., Lounibos, L.P. 2015b. Satyrization without evidence
of successful insemination from interspecific mating between invasive mosquitoes. Biol. Lett. 11(9),
20150527.
Nasci et al. (1989) and Bargielowski et al. (2013) both showed a higher rate of cross insemination in cages between *Ae. aegypti* females and *Ae. albopictus* males than in the reverse cross, which supports asymmetric reproductive interference as a mechanism for competitive displacements of *Ae. aegypti*. Leahy & Craig (1965) and Tripet et al. (2011) showed that injections of heterologous male accessory gland substances triggered the switch to post-mating behaviour in *Ae. aegypti* females, but not in *Ae. albopictus* females, in the same way that conspecific male accessory gland substances stimulate oviposition (Leahy and Craig, 1965) and refractoriness to mating (Tripet et al., 2011). Here I show that *Ae. aegypti* females become refractory to further mating after exposure to *Ae. albopictus* males, even if heterospecific sperm are not deposited in their spermathecae.

**Methods**

Experiments, each replicated three times within the same cohort, used second to fifth generation *Ae. aegypti* collected from Key West, FL, USA and *Ae. albopictus* from Vero Beach, Florida, USA.

**Absence of Heterospecific Sperm in Spermathecae and Refractoriness to Mating**

In each replicate, an interspecific–conspecific treatment was paired with a sham-treated control. In the interspecific–conspecific treatment, 3-day-old virgin *Ae. aegypti* females (n = 150) were housed in the same cage with 3-day-old *Ae. albopictus* males (n = 150) for three weeks. Detection of the presence or the absence of sperm in the spermathecae of live females was accomplished with the technique explained in chapter 2. Each female that was negative for heterospecific sperm was exposed to two 3-day-old virgin *Ae. aegypti* males for 24 h in the same cage. After exposures to conspecifics, healthy females were dissected to check for sperm in the spermathecae.
(44–50 females per replicate). In the sham-treated control, a ‘sham’ sperm check was applied to 24-day-old virgin *Ae. aegypti* females that were subsequently housed with 3-day-old conspecific males, and dissected (20–23 females per replicate).

A t-test was performed to analyse differences in the proportions (proportions transformed by Freeman–Tukey method) of females inseminated by conspecifics between the interspecific–conspecific treatment and the sham-treated control using SPSS Statistics 21.

**Transfer of Semen**

Females were exposed to males labelled with stable isotopes, in order to detect semen transfer. Mosquitoes were labeled with $^{15}$N-glycine (NLM-202-1; Cambridge Isotope Laboratories, Inc.), following procedures described by Helinski et al. (2012), with some modifications. To obtain males labeled with $^{15}$N, eggs were hatched in a vacuum, and 200 first instar larvae were placed in a tray with 1 liter of Milli-Q water. Larvae received 68 mg of $^{15}$N-glycine on day 1 and 20 mg on day 3 (25% enrichment based on nitrogen content in yeast and lactalbumin). Larvae also received a standard diet (a 1:1 mixture of lactalbumin and yeast) according to the following schedule: day one: 38 mg, day three: 75 mg, day four: 113 mg, day five: 150 mg, day six: 113 mg. To obtain unlabeled females, larvae were reared with standard diet (a 1:1 mixture of lactalbumin and yeast).

In each replicate, an interspecific treatment, a positive control and a control were performed. In the interspecific treatment, 3-day-old virgin *Ae. aegypti* females ($n = 150$) were exposed to 3-day-old $^{15}$N-labelled *Ae. albopictus* males ($n = 150$) in a cage for three weeks. In the positive control, 3-day-old virgin *Ae. aegypti* females ($n=150$) were exposed to $^{15}$N-labelled *Ae. aegypti* males ($n=150$) for three weeks; and in the control, a
cage with 3-day-old unlabelled females (n=150) was set. After three weeks, the presence or the absence of sperm in spermathecae was checked in live females, as explained in chapter 2. After the sperm check, females were frozen at 22°C for 30 min, and dried overnight at 55°C. Stable isotope analysis in whole female bodies was performed by the Light Stable Isotope Mass Spectrometry Laboratory in the Department of Geological Sciences at the University of Florida.

**Data analysis.** Nitrogen isotopic results are expressed in standard delta notation relative to air (Hübschmann, 2009). A female was considered labeled when her delta value was above a conservative threshold (Helinski et al., 2007; 2008; 2012; Macneale et al., 2005). This threshold was defined as three standard deviations above the mean of the reference value, based on measurements of unlabeled females. Each of the replicates with its controls was performed at a different time, with separate threshold values established and applied for each replicate.

**Refractoriness to Conspecific Mating Assessed Through Egg Viability**

This experiment was performed before the technique for sperm detection in live *Ae. aegypti* females was developed. In each replicate, an interspecific–conspecific treatment was paired with a control. In the interspecific–conspecific treatment, 3-day-old virgin *Ae. aegypti* females (n = 150) were exposed to 3-day-old *Ae. albopictus* males (n = 150). After three weeks, *Ae. albopictus* males were removed; 50% of the females were dissected to determine sperm presence in spermathecae (52–56 females per replicate); the remaining females were kept in the cage for 24 h. Subsequently, females were exposed for 3 days to 3-day-old conspecific males in a 1:1 ratio and were allowed to blood feed from a live chicken according to UF-IACUC Protocol no. 201003892. Each blood fed female was transferred to an individual cage with an oviposition site. Eggs
were collected from each female (18–29 females per replicate), counted and hatched to determine if a female laid viable or non-viable eggs. Production of viable eggs was used as a proxy for successful intraspecific insemination. In the controls, 24-day old virgin *Ae. aegypti* females were exposed to 3-day-old conspecific males for 3 days and subsequently allowed to blood feed. Each blood fed female was allowed to oviposit (13-18 females per replicate).

To test differences between the actual proportion of *Ae. aegypti* females that laid viable eggs in the interspecific–conspecific treatment versus the expected proportion based on the females that had no heterospecific sperm in spermathecae, a $\chi^2$-goodness of fit analysis was performed using SPSS Statistics 21. To test differences in the number of eggs laid between the interspecific–conspecific treatment and the control, a t-test was performed using SPSS Statistics 21.

**Results**

**Absence of Heterospecific Sperm in Spermathecae and Refractoriness to Mating**

An arithmetic average of 49.1+1.6 (s.d.)% of *Ae. aegypti* females were negative for sperm in spermathecae after exposures to *Ae. albopictus* males. These females were exposed to *Ae. aegypti* males. An arithmetic average of 69.7+6.2 (s.d.)% of the pre-exposed females mated successfully with conspecifics. Significantly more control females that were exposed to conspecifics mated successfully, an arithmetic average of 96.9+2.7(s.d.)% ($t_4 = 26.0$, $P = 0.004$). These results show that many *Ae. aegypti* females that had no *Ae. albopictus* sperm in their spermathecae were refractory to subsequent mating with conspecifics.
Transfer of Semen

All Ae. aegypti females positive for sperm in spermathecae and approximately half of the females whose spermathecae were empty after exposures to Ae. albopictus males were labelled with $^{15}$N (Figure 3-1). Our results suggest that ejaculate from Ae. albopictus males was transferred to many females without deposition of sperm in spermathecae.

Refractoriness to Conspecific Mating Assessed Through Egg Viability

An arithmetic average of 46.7+6.8 (s.d.)% of dissected Ae. aegypti females that were exposed to Ae. albopictus males did not have heterospecific sperm in their spermathecae. Egg viability was assessed for the remaining females of the cage that were subsequently exposed to conspecifics, blood fed and allowed to oviposit. A mean of 16.3+6.9 (s.d.)% of these females laid viable eggs, which is significantly fewer than expected based on the proportion of females that had no heterospecific sperm in their spermathecae ($X^2_{(1)} = 27:3, P < 0.001$). All control females laid viable eggs. These results indicate that many females without heterospecific sperm in their spermathecae could not mate with conspecifics as evidenced by inviable eggs.

The arithmetic average number of eggs laid by Ae. aegypti females that were exposed to Ae. albopictus males and subsequently to Ae. aegypti males was 85.7+17.6 (s.d.), which is not significantly different from control females (93.1+6.8 (s.d.)) ($t_4 = 20.683, P = 0.532$).

Discussion

Models of competitive interactions of two species have shown that low levels of reproductive interference, coupled with resource competition, can lead to local extinctions and displacements (Kishi and Nakazawa, 2013; Ribero, 1988; Ribeiro and
Spielman, 1986). By showing that analyses of spermathecal contents may underestimate interspecific matings, my results strengthen the importance of reproductive interference as a mechanism to explain the rapid competitive displacements of *Ae. aegypti* by *Ae. albopictus* that have been documented in the southeastern USA and Bermuda (Kaplan et al., 2010; Lounibos, 2002).

In culicine mosquitoes, insemination is defined as the deposition of semen (sperm and secretions of male accessory glands) in the female’s bursa and the subsequent passage of sperm into the spermathecae (Clements, 1999). My results show that satyrization without successful insemination occurred in many *Ae. aegypti* females and that these females were refractory to subsequent mating with conspecifics even without *Ae. albopictus* sperm in their spermathecae. Transfer of semen, including male accessory gland substances, is likely rendering females refractory to further mating, regardless of the presence of sperm in their spermathecae. This is supported by the experiment in which females were exposed to $^{15}$N-labelled *Ae. albopictus* males and approximately half of the females without sperm in spermathecae were labelled with $^{15}$N. Additional evidence for satyrization without successful insemination was provided by the experiment that assessed refractoriness to mating through egg viability. Compared with the higher proportion of females that had no heterospecific sperm in their spermathecae, only a low percentage of females actually laid viable eggs. This suggests that many females that were negative for heterospecific sperm in the spermathecae were refractory to further mating with conspecifics and, hence, laid inviable eggs.
I hypothesize that male accessory gland products and sperm from *Ae. albopictus* are transferred to the female bursa, but not to the spermathecae, in those *Ae. aegypti* with no sperm in their spermathecae that were unreceptive to further mating with conspecifics. Following deposition of semen in the female mosquito bursa, sperm ordinarily reach the spermathecal vestibule by their own motility and then are carried through the spermathecal ducts to the spermathecae by actions of the female (Clements, 1999). As heterospecific mating occurs by mistake owing to failure in species recognition (Gröning and Hochkirch, 2008), it appears that sperm from *Ae. albopictus* are not always transferred properly to *Ae. aegypti* spermathecae. In one live sperm check, the bursa was expelled from the female and seen to harbour semen even though the spermathecae had no sperm (Figure 3-2).

Many females that had no sperm in their spermathecae and were labelled with $^{15}$N tended to have lower values of $^{15}$N compared with the females that were positive for sperm. A possible explanation is that in the bursa, sperm are not stored or maintained viable for prolonged periods as occurs in the spermathecae. In intraspecific matings, the sperm that do not reach the spermathecae and remain in the bursa lose their motility two days after coitus (Clements, 1999). If nonmotile sperm begin to degrade in the bursa, it is probable that lower values of $^{15}$N would be detected.

In previous studies on interspecific matings in insects, failures in sperm storage, or retention in the female’s reproductive tract have been noted (Leahy and Craig, 1967; Nasci et al., 1989; Patterson, 1947; Vick, 1973). In interspecific matings between *Ae. aegypti* and *Ae. albopictus*, Nasci et al. (1989) observed that most *Ae. aegypti* females inseminated by *Ae. albopictus* males had few dead sperm in their spermathecae, and
Leahy & Craig (1967) detected a high frequency of immobilized sperm in the spermathecae of *Ae. aegypti* females. In crosses between *Drosophila hamatofila* females and *D. mojavensis* males, Patterson (1947) reported that most of the sperm that were stored in the ventral receptacle after copulation ‘disappeared altogether’, as confirmed by later dissections. In interspecific matings between the dermestid beetles *Trogoderma glabrum* and *T. inclusum*, males transfer spermatophores to *T. glabrum* females, but sperm usually do not reach the spermathecae (Vick, 1973). This example resembles the pattern of reproductive interference that occurs between *Ae. aegypti* and *Ae. albopictus*, in that refractoriness to mating with conspecifics and oviposition of sterile eggs occurs when *T. glabrum* females copulate with *T. inclusum* males, but not in the reverse cross. Vick (1973) suggested that transfer of male accessory gland material may occur, independent of the transfer of sperm to the spermathecae.

My results demonstrate that reproductive interference may also occur, even if *Ae. albopictus* sperm are not transferred to *Ae. aegypti* spermathecae. Therefore, the low frequency of interspecific mating that has been found in wild-caught females, measured as percentage of females with heterospecific sperm in their spermathecae (Tripet et al., 2011), can underestimate the incidence of reproductive interference in nature.
Figure 3-1. $\delta^{15}$N values of *Ae. aegypti* females exposed to *Ae. albopictus* males and control females. Each dot represents a female mosquito. Females negative for heterospecific sperm in spermathecae (filled circles), females positive for heterospecific sperm in spermathecae (filled triangles), control females (filled diamonds), and positive control females (x). Females above the threshold (dotted line) are considered labeled with $^{15}$N.
Figure 3-2. Female with semen in the bursa and empty spermathecae. A) *Aedes aegypti* bursa contains *Ae. albopictus* semen yet the spermathecae are empty. B) Dissected terminalia of the same female showing sperm in the dissected bursa and the empty spermathecae. Photo courtesy of María Cristina Carrasquilla Ferro.
CHAPTER 4
SPERMATHECAL USE IN *Aedes aegypti* AND *Aedes albopictus*

**Introduction**

In many animal species that have internal fertilization, with the exception of most mammals, females may store sperm, transferred by males during mating, for prolonged periods of time (Pitnick et al., 1999). Stored sperm are held internally in the females' reproductive tract, where they are kept viable, and are released when fertilization of the ovum or egg takes place (Birkhead and Moller, 1992; Davey, 1965; Degner and Harrington, 2016). For example, different organs are used to store sperm in some or most of the species of the following groups: the uterus, the utero-tubal junction and the oviduct in bats (Racey, 1979); the sperm storage tubules in birds and reptiles (Birkhead and Moller, 1992; Fox, 1963); the spermathecae in some arthropods (Glesson, 1990) and amphibians (Sever et al., 2004); and the seminal receptacle in other arthropods (Pitnick et al., 1999; Waddy and Aiken, 1990).

Females of most insects store and maintain sperm internally in the spermathecae (Gullan and Cranston, 2010; Simmons, 2001). Most insect species have a single spermatheca (Gullan and Cranston, 2010; McAlpine et al., 1981), but the number of spermathecae varies from one to three (Mc.Alpine et al., 1981). There is a report of one exceptional insect species, the earwig *Diplatys macrocephalus*, that has ten spermathecae (Popham, 1965).

In different mosquito species the number of spermathecae varies from one to three (Downes, 1968; Clements, 1999). The genera *Anopheles*, *Uranotaenia*, *Aedeomyia*, some *Ficalbia*, and certain species of *Aedes* have a single spermatheca, while in the genus *Mansonia*, the subgenus *Mansonioides*, two or one of the three
spermathecae may be very small and difficult to detect. The genus *Hodgesia* has three, of which the third may be minute. The genera *Culex*, *Toxorhynchites*, and most aedine mosquitoes have three spermathecae (Edwards, 1941; Neveu-Lemaire, 1902). *Aedes aegypti* and *Ae. albopictus* have one large, medial and two smaller, lateral spermathecae (Clements, 1999). Although mosquitoes are generally considered to be monandrous (Clements, 1999), some mosquito species have spermathecal features associated with polyandry and others do not (Yuval, 2006).

Multiple spermathecae have been associated with cryptic female choice in polyandrous insects, allowing females to discriminate between sperm of different mates (Eberhard, 1996; Hellriegel and Ward, 1998; Yuval, 2006). The influence of females on the process of sperm storage has been studied in yellow dung flies *Scathophaga stercoraria* that are polyandrous and have a singlet and a doublet spermatheca. In single matings, the number of spermathecae with sperm is influenced by male size and amount of stored sperm (Ward, 1993). Double-mated females are able to differentially store sperm of their mates. Sperm storage may be influenced by a female’s body size, the size of the second male, and the relative sizes of the two males (Ward, 1993), suggesting that multiple spermathecae allow females to have a better control of offspring paternity (Hellriegel and Bernascon, 2000; Otronen et al., 1997; Ward, 1993; 1998). However, other authors argue that non-random paternity in yellow dung flies is explained solely by sperm competition (Simmons et al., 1996).

In the mosquitoes *Ae. aegypti* and *Ae. albopictus* there is scanty information about spermathecal usage. Studies on spermathecal filling in *Ae. aegypti* have found that 8% of the females store sperm in two spermathecae and 92% in all three
spermathecae. In females containing sperm in three spermathecae, the central and one of the lateral spermathecae store numerous sperm, and the second lateral spermatheca has few sperm (Jones and Wheeler, 1965). For *Ae. albopictus* it has been reported that females store sperm mainly in two spermathecae (Oliva et al., 2013). For wild-caught females, Tripet et al. (2011) found that most *Ae. aegypti* and *Ae. albopictus* females had sperm in two spermathecae. Interspecific matings that occur between these two species may be associated with multiple inseminations. Molecular analysis of sperm in spermathecae of wild-caught females, has shown either mixed sperm of both species in one spermatheca, or segregation of conspecific and heterospecific sperm in different spermathecae (Tripet et al., 2011).

*Aedes aegypti* and *Ae. albopictus* are ecologically similar species (Murrell et al., 2011), that have evolved independently (Leahy and Craig, 1967). They belong to the subgenus *Stegomyia*, and were originally confined to tropical and subtropical regions of the Old World. *Aedes aegypti* belongs to the *Aegypti* Group native to the Afrotropical Region (Huang, 2004), while *Ae. albopictus* is part of the *Scutellaris* Group, originally from Southeast Asia and Pacific Islands (Huang, 1972). Differences in the mating biology of *Ae. aegypti* and *Ae. albopictus* were detected by De Jesus and Reiskind (2016). In intraspecific matings more sperm are stored in spermathecae of *Ae. albopictus* compared with *Ae. aegypti*, and fecundity increases were observed in *Ae. albopictus* females, but not in *Ae. aegypti*, that mate with larger males. Differences were not found between *Ae. aegypti* and *Ae. albopictus* females that mate with large or small males in the number of sperm stored in spermathecae. However, in *Ae. aegypti* the number of sperm in spermathecae declined in gonotrophic cycles three and four. Using
females reared in a moderate density larval environment, De Jesus & Reiskind (2016) showed that female size and number of sperm stored in *Ae. aegypti* or *Ae. albopictus* spermathecae are not correlated.

Postcopulatory female control of sperm storage has been implied in mosquitoes, as sperm arrive by their own motility from the female bursa to the spermathecal vestibule, and then are transported through the spermathecal ducts by actions of the female (Clements, 1999; Degner and Harrington, 2016). On the other hand, male influence on female behavior and physiology can be pronounced, especially through the post-copulatory actions of male accessory gland substances, which differ prominently between these species (Boes et al., 2014) and have different effects after interspecific matings (Bargielowski and Lounibos, 2016; Tripet et al., 2011). In this study I examined the influence of mosquito body size on spermathecal usage in *Ae. aegypti* and *Ae. albopictus*, after intraspecific and interspecific matings in light of the evidence of different reproductive biologies of these species and differential effects of their male accessory gland products in cross-matings. As heterologous male accessory gland proteins stimulate oviposition (Leahy and Craig, 1965), and refractoriness to further mating (Tripet et al., 2011) in *Ae. aegypti* females, but not in *Ae. albopictus* females, and larger *Ae. albopictus* males may transfer more seminal fluid substances during mating and induce in females more pronounced post-mating responses (De Jesus and Reiskind, 2016), I hypothesized that *Ae. aegypti* and *Ae. albopictus* females inseminated by large *Ae. albopictus* males would have more spermathecae containing sperm than crosses involving *Ae. aegypti* males. My experimental design also allowed
me to quantify how mosquito size affects insemination rates in these two mosquito species.

**Methods**

*Aedes aegypti* were satyrization-susceptible $F_{10}$-$F_{11}$ progeny from collections in Key West, Florida (Bargielowski et al., 2013). *Aedes albopictus* were $F_{11}$-$F_{12}$ progeny from Vero Beach, Florida. Mosquitoes were maintained in insectaries at the Florida Medical Entomology Laboratory at 26.78 ± 0.87˚C, 89.18 ± 9.57% RH, and 26.99 ± 0.28˚C, 80.72 ± 4.67% RH, and 14L:10D day length.

Eggs were hatched in a vacuum and first instar larvae were placed in a tray with 1L of water. To produce differences in adult body size, larvae were reared in groups of different numbers (75 per tray for large mosquitoes and 500 for small mosquitoes). Food (1:1 yeast: lactalbumin) was added to each tray in the following schedule: 75, 38, 75, 113, 150 mg on day: 1, 3, 4, 5, and 6, respectively (Ponlawat and Harrington, 2007). Pupae were sexed based on differences in terminalia morphology, and put in containers with no more than 20 individuals in order to confirm the sex of adults, before setting up the cage crosses. If a mistake in sexing was detected, the container was discarded (Bargielowski et al., 2013). Intraspecific and interspecific crosses were set up, exposing large or small *Ae. aegypti* or *Ae. albopictus* females to large or small conspecific or heterospecific males. The following crosses were performed: large females x large males, large females x small males, small females x large males, small females x small males. As two intraspecific crosses (*Aedes aegypti* females x *Ae. aegypti* males, and *Ae. albopictus* females x *Ae. albopictus* males) and two interspecific crosses (*Aedes aegypti* females x *Ae. albopictus* males, and *Ae. albopictus* females x *Ae. aegypti* males) were set up, in total 16 different combinations were performed. Each cross
combination was replicated in three sequential repetitions. In each cross, 150 three day old females were caged with 150 three day old males for three weeks in Bug Dorm™ cages (30 by 30 by 30 cm). Sugar solution (10%) was constantly provided.

Females were dissected three weeks after exposure. Females were anesthetized with chloroform and spermathecal dissections were performed in water immediately thereafter under a dissecting microscope. Spermathecae were observed under a phase contrast microscope. To be able to see sperm moving inside the spermathecae, no more than three females were anesthetized and dissected at the same time, as sperm stop moving a few minutes after dissection. Observation of sperm movement helped to assess if a spermatheca contained sperm, particularly when few were present. The insemination status of each female was recorded. A female was considered inseminated if she had sperm in any of the three spermathecae. For inseminated females the number of spermathecae that were positive for sperm (1, 2 or 3), and their location: central (large), and/or one or two of the lateral (smaller) spermathecae were recorded.

Female and male mosquitoes from the same developmental cohorts, that were not used for the cage crosses, were dried in aluminum cups for 24 hours at 55˚C in an oven (Fisher Isotemp) and weighed individually in a Orion Cahn C-33 microbalance (Thermo Scientific).

Statistical analysis: A logistic linear model using Proc GLIMMIX (SAS, 2011) was run, to analyze the effect of cross type (Ae. aegypti ♀ x Ae. albopictus ♂, Ae. albopictus ♀ x Ae. aegypti ♂), female size (large, small), and male size (large, small) on the proportion of inseminated females.
The average number of spermathecae filled with sperm in inseminated females was analyzed as a full factorial linear fixed effects model with three fixed categorical effects, cross-type, female size, and male size using Proc GLIMMIX (SAS Inc.). Separate analyses were run for the Ae. aegypti female crosses (Aedes aegypti ♀ x Ae. aegypti ♂, Ae. aegypti ♀ x Ae. albopictus ♂) and for Ae. albopictus female crosses (Aedes albopictus ♀ x Ae. albopictus ♂, Ae. albopictus ♀ x Ae. aegypti ♂) to test for species-specific responses. Differences in mosquito dried body mass were analyzed with ANOVA (SPSS Statistics 21).

Results

Mosquito Mass

Small and large females (F<sub>3, 185</sub> = 162.57, P < .0001) and males (F<sub>3, 195</sub> = 43.21, P < .0001) differed significantly in dried body mass (Table 4-1, 4-2). However, interspecific comparisons of body masses between individuals of the same size class were non-significant (Table 4-2).

Table 4-1. Average dried body mass of large and small Ae. aegypti and Ae. albopictus females and males.

<table>
<thead>
<tr>
<th></th>
<th>Females (mg ± sd)</th>
<th>n</th>
<th>Males (mg ± sd)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Ae. aegypti</td>
<td>1.505 ± 0.304</td>
<td>45</td>
<td>0.587 ± 0.102</td>
<td>45</td>
</tr>
<tr>
<td>Large Ae. albopictus</td>
<td>1.451 ± 0.246</td>
<td>45</td>
<td>0.578 ± 0.138</td>
<td>45</td>
</tr>
<tr>
<td>Small Ae. aegypti</td>
<td>0.742 ± 0.168</td>
<td>50</td>
<td>0.391 ± 0.088</td>
<td>54</td>
</tr>
<tr>
<td>Small Ae. albopictus</td>
<td>0.745 ± 0.178</td>
<td>49</td>
<td>0.431 ± 0.099</td>
<td>55</td>
</tr>
</tbody>
</table>
### Table 4-2. P values after Tukey multiple comparisons of dried body mass.

<table>
<thead>
<tr>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pair comparisons</strong></td>
<td><strong>Pair comparisons</strong></td>
</tr>
<tr>
<td>Large vs. Small <em>Ae. aegypti</em> ♀</td>
<td>Large vs. Small <em>Ae. aegypti</em> ♂</td>
</tr>
<tr>
<td>Large vs. Small <em>Ae. albopictus</em> ♀</td>
<td>Large vs. Small <em>Ae. aegypti</em> ♂ vs. Large <em>Ae. albopictus</em> ♂</td>
</tr>
<tr>
<td>Large <em>Ae. aegypti</em> ♀ vs. Large <em>Ae. albopictus</em> ♀</td>
<td>Large <em>Ae. aegypti</em> ♂ vs. Large <em>Ae. albopictus</em> ♂</td>
</tr>
<tr>
<td>Small <em>Ae. aegypti</em> ♀ vs. Small <em>Ae. albopictus</em> ♀</td>
<td>Small <em>Ae. aegypti</em> ♂ vs. Small <em>Ae. albopictus</em> ♂</td>
</tr>
</tbody>
</table>

### Insemination Rate

For the analysis of insemination rate I only included the interspecific crosses (*Ae. aegypti* ♀ x *Ae. albopictus* ♂, and *Ae. albopictus* ♀ x *Ae. aegypti* ♂), as in the intraspecific crosses (*Ae. aegypti* ♀ x *Ae. aegypti* ♂, and *Ae. albopictus* ♀ x *Ae. albopictus* ♂) proportions of inseminated females were above 0.99. A significant three-way interaction was found between type of mating (*Ae. aegypti* ♀ x *Ae. albopictus* ♂ and *Ae. albopictus* ♀ x *Ae. aegypti* ♂), female size, and male size (Table 4-3). Owing to the much higher insemination rates in interspecific crosses of *Ae. aegypti* females, there was a much broader range in mean proportions inseminated compared to the converse crosses involving *Ae. albopictus* females (Figure 4-1). Curiously, the highest insemination rates of *Ae. aegypti* females were observed in the cross of large individuals of both sexes, but this pattern did not apply to *Ae. albopictus* females, where the large x large cross did not produce a significantly different mean proportion inseminated from other crosses. Most, but not all, interspecific insemination rates were significantly higher in crosses between *Ae. aegypti* females and *Ae. albopictus* males. There were no significant differences in insemination rates between the cross *Ae.
*aegypti* small female x *Ae. albopictus* large male and the cross *Ae. albopictus* small female x *Ae. aegypti* large male (Figure 4-1).

Table 4-3. Effects on proportions inseminated of cross type (*Aedes aegypti* ♀ x *Ae. albopictus* ♂, and *Ae. albopictus* ♀ x *Ae. aegypti* ♂), male and female size, and interactions.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross</td>
<td>1</td>
<td>16</td>
<td>247.18</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Female size</td>
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<td>16</td>
<td>29.66</td>
<td>&lt; .0001</td>
</tr>
<tr>
<td>Cross* Female size</td>
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<td>16</td>
<td>19.69</td>
<td>0.0004</td>
</tr>
<tr>
<td>Male size</td>
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<td>16</td>
<td>19.65</td>
<td>0.0004</td>
</tr>
<tr>
<td>Cross* Male size</td>
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<td>4.38</td>
<td>0.0525</td>
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<tr>
<td>Female size * Male size</td>
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<td>16</td>
<td>0.43</td>
<td>0.5191</td>
</tr>
<tr>
<td>Cross* Female size * Male size</td>
<td>1</td>
<td>16</td>
<td>29.11</td>
<td>&lt; .0001</td>
</tr>
</tbody>
</table>

**Spermathecal Use**

**Sperm location**

Overall, 1,801 females had sperm in three spermathecae, 1,865 had sperm in two spermathecae and 53 had sperm in one spermatheca. In females containing sperm in two spermathecae, the sperm were located in the central and in one of the lateral spermathecae in 99.95% of the mosquitoes, and in the two lateral spermathecae in 0.05% of the females. In the mosquitoes that had sperm in one spermatheca, sperm were found in the central spermatheca in 94.33% of the females, and in one of the lateral spermathecae in 5.66% of the individuals.

**Average number of spermathecae used**

*Aedes aegypti* female crosses: In intraspecific matings between *Ae. aegypti* females and *Ae. aegypti* males, the average number of spermathecae used was 2.43 and in matings between *Ae. aegypti* females and *Ae. albopictus* males the average number of spermathecae used was 2.36. No significant differences were found between cross type (*Ae. aegypti* ♀ x *Ae. aegypti* ♂ and *Ae. aegypti* ♀ x *Ae. albopictus* ♂), female size, or male size (Figure 4-2). Interactions were not significant (Table 4-4).
**Aedes albopictus** female crosses: In intraspecific matings between **Ae. albopictus** females and **Ae. albopictus** males, the average number of spermathecae used was 2.57 and in matings between **Ae. albopictus** females and **Ae. aegypti** males the average number of spermathecae used was 2.56. There was a significant effect of female size, as larger females had a higher mean number of spermathecae filled (2.67) than smaller (2.46). No significant differences were found between cross type (**Ae. albopictus** ♀ x **Ae. albopictus** ♂ and **Ae. albopictus** ♀ x **Ae. aegypti** ♂), or male size (Figure 4-2).

Interactions were not significant (Table 4-5).

**Table 4-4.** Effects on average number of spermathecae containing sperm of cross type (**Aedes aegypti** ♀ x **Ae. aegypti** ♂, and **Ae. aegypti** ♀ x **Ae. albopictus** ♂), male and female size, and interactions.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
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</tr>
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<tbody>
<tr>
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<td>0.2855</td>
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<tr>
<td>Female size</td>
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<tr>
<td>Cross * Female size</td>
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<td>16</td>
<td>0.02</td>
<td>0.9018</td>
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<tr>
<td>Male size</td>
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<td>0.7977</td>
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<tr>
<td>Cross * Male size</td>
<td>1</td>
<td>16</td>
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<td>0.6848</td>
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<tr>
<td>Female size * Male size</td>
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<tr>
<td>Cross * Female size * Male size</td>
<td>1</td>
<td>16</td>
<td>0.71</td>
<td>0.4124</td>
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</table>

**Table 4-5.** Effects on average number of spermathecae containing sperm of cross type (**Aedes albopictus** ♀ x **Ae. albopictus** ♂, and **Ae. albopictus** ♀ x **Ae. aegypti** ♂), male and female size, and interactions.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num DF</th>
<th>Den DF</th>
<th>F value</th>
<th>P</th>
</tr>
</thead>
<tbody>
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<td>0.8856</td>
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<td>Female size</td>
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<td>16</td>
<td>6.02</td>
<td>0.0260</td>
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<tr>
<td>Cross * Female size</td>
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<td>0.1953</td>
</tr>
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<td>Male size</td>
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<td>0.25</td>
<td>0.6242</td>
</tr>
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<td>Cross * Male size</td>
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<td>16</td>
<td>0.35</td>
<td>0.5646</td>
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<td>Female size * Male size</td>
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<td>0.01</td>
<td>0.9173</td>
</tr>
<tr>
<td>Cross * Female size * Male size</td>
<td>1</td>
<td>16</td>
<td>0.97</td>
<td>0.3405</td>
</tr>
</tbody>
</table>

**Discussion**

Analysis of the proportion of inseminated females reveals an interaction between type of mating (**Aedes aegypti** females x **Ae. albopictus** males, **Ae. albopictus** females x...
Ae. aegypti males), female size and male size. Interspecific insemination is higher in most of the crosses between Ae. aegypti females and Ae. albopictus males compared with crosses between Ae. albopictus females and Ae. aegypti males, as has been shown in previous studies that did not sort mosquitoes by size (Bargielowski et al., 2013; Nasci et al., 1989). In my study, the cross Ae. aegypti large female x Ae. albopictus large male had the highest proportion of insemination among interspecific pairings, and three of the Ae. albopictus female x Ae. aegypti male crosses (large female x large male, large female x small male, small female x small male) had the lowest proportions of insemination. However, not all interspecific insemination rates were significantly higher between Ae. aegypti females and Ae. albopictus males. Significant differences were not found between the cross Ae. aegypti small female x Ae. albopictus large male and the cross Ae. albopictus small female x Ae. aegypti large male.

My analysis of the average number of spermathecae used in crosses with Ae. albopictus females shows that larger females have more spermathecae containing sperm compared with smaller ones, and that in crosses with Ae. aegypti females, there is no effect of female body size on the average number of spermathecae positive for sperm. Many studies have documented a positive correlation between female body size and factors related with reproductive success in other mosquito species, including fecundity (Hawley, 1985; Okanda et al., 2002; Renshaw et al., 1994), insemination (Okanda et al., 2002) and parity rate (Renshaw et al., 1994). However, it has also been reported that female size does not influence mating probability (Charlwood et al., 2003). Particularly, for Ae. aegypti and Ae. albopictus, it has been documented that larger
females lay more eggs (Blackmore and Lord, 2000; Briegel et al., 1990). De Jesus and Reiskind (2016) found no correlation between female size and number of stored sperm for either *Ae. aegypti* and *Ae. albopictus*. However, they did not generate large and small females, they produced various sized females by rearing larvae in intermediate crowding conditions. In my study, female size significantly affected the number of spermathecae with sperm in *Ae. albopictus*, but not in *Ae. aegypti*, which implies differences in the mating biology of these two species.

I did not see an effect of male size on the number of spermathecae containing sperm. In *Ae. aegypti*, the independence of the size of the male and the effects on post mating behaviors of the female has been shown by Dickinson and Klowden (1997). In their experiments, small males reared on a low quality diet have less protein in their male accessory glands and use less protein during mating compared to large males reared on a high quality diet. However, both large and small males induced in females refractoriness to further mating. In a different experiment, it was shown that in *Ae. aegypti*, the effect on female post-mating behavior was modulated by the nutritional state of the adult male. Starved males have less total protein in their male accessory glands, utilize fewer proteins during mating, and are less efficient at inhibiting the host-seeking behavior of the gravid females that they mated than sugar-fed males (Fernández and Klowden, 1995). De Jesus and Reiskind (2016), found an effect of male body size on fecundity of *Ae. albopictus*, but not of *Ae. aegypti*. These authors suggested that larger *Ae. albopictus* males transfer more seminal fluid products to females. In my experiment, I generated large and small mosquitoes, rearing the larvae under different crowding conditions, but using the same type of food. In future
experiments, it would be interesting to generate large and small individuals, also altering the quality of the larval food (Dickinson and Klowden, 1997), and to vary the nutritional state of the adult male (Fernández and Klowden, 1995) in order to understand better the effect of male quality on spermathecal usage in *Ae. aegypti* and *Ae. albopictus*.

In my experiments, there was no evidence of an effect of cross-type on the number of spermathecae containing sperm. It has been shown that injections of heterologous male accessory gland substances influence the switch to post-mating behavior in *Ae. aegypti* females, but not in *Ae. albopictus*, in the same way that conspecific male accessory gland substances trigger oviposition (Leahy and Craig, 1965) and refractoriness to mating (Tripet et al. 2011), so other post-mating processes that are influenced by male accessory gland proteins could have a similar response in interspecific crosses between *Ae. aegypti* females and *Ae. albopictus* males. However, as suggested in chapter 3, in some *Ae. aegypti* females exposed to *Ae. albopictus* males, sperm storage doesn’t occur properly as male accessory gland products and sperm may be transferred to the female bursa, but not to the spermathecae (Carrasquilla and Lounibos, 2015b), and some authors have seen dead or immobilized sperm in spermathecae (Leahy and Craig, 1967; Nasci et al., 1989). My results for heterospecific crosses between *Ae. albopictus* females and *Ae. aegypti* males show that in the relatively few females that are inseminated (10.52%), patterns of sperm storage are similar to the ones found in intraspecific matings. In crosses between *Ae. albopictus* females and *Ae. aegypti* males, it has also been reported the presence of dead sperm in spermathecae, but not as frequent as in interspecific crosses between *Ae. aegypti* females and *Ae. albopictus* males (Leahy and Craig, 1967). Even though I
was not studying sperm viability when I was assessing if spermathecae had sperm, I could see that sperm were moving. Thus in both interspecific crosses, females that are inseminated have similar patterns of sperm storage and maintenance compared to conspecific matings. However, further studies will be necessary to understand the specific mechanisms behind this process. In intraspecific matings, it is broadly known that sperm storage is influenced by sperm locomotion and active transport of the female reproductive tract (Clements, 1999; Degner and Harrington, 2016). Male accessory gland substances transferred during mating may play a role in sperm localization in the spermathecae and sperm storage, as it has been shown in anopheline mosquitoes (Rogers et al., 2009) and Drosophila (Avila and Wolfner, 2009; Avila et al., 2011; Ram and Wolfner, 2009).

In order to better understand the influence of the female on spermathecal usage in Ae. aegypti and Ae. albopictus it would interesting to observe patterns of spermathecal filling, right after mating and sometime after mating in females that have been kept awake and in mosquitoes that have been anesthetized. In yellow dung flies S. stercoraria, which are polyandrous, Ward (1998) found that in individuals that were dissected immediately after first copula, larger females have more sperm in their spermathecae than smaller ones. In females that were anaesthetized for 30 minutes, larger females had even more sperm in the spermathecae, showing that sperm are able to move on their own in the female reproductive tract. However, if females were kept awake for 30 minutes after dissection, the relationship was inverse, and larger females had less sperm in their spermathecae than smaller (Ward, 1998). This author proposed
that larger females may be more selective than smaller ones, about the particular sperm that they store.

My results show that larger \textit{Ae. albopictus} females have more spermathecae containing sperm compared with smaller females, and that there is no effect of \textit{Ae. aegypti} female body size on the average number of spermathecae used. I did not find an effect of male size or cross-type. The different effect that female size has on spermathecal usage in \textit{Ae. aegypti} and \textit{Ae. albopictus} provides more evidence that there are differences in the mating biology of these two species. Further studies are necessary to have a better understanding on spermathecal usage in these two mosquito species.
Figure 4-1. Mean proportion of inseminated females in eight interspecific crosses. Same letter indicates no significant difference.
Figure 4-2. Average number of spermathecae containing sperm. Number of ♀ with spermathecae positive for sperm in each cross is shown above the error bar.
CHAPTER 5
CONCLUSIONS

My work provides evidence of the importance of satyrization as a mechanism to explain competitive displacement of *Ae. aegypti* by *Ae. albopictus*. Previously, low rates of cross-matings (1.12-3.73%), measured as percentage of females with interspecific sperm in their spermathecae, between these two mosquito species were detected in nature (Bargielowski et al., 2015a; Tripet et al., 2011). My results showed that satyrization without successful insemination of *Ae. aegypti* by *Ae. albopictus* may occur, suggesting that frequencies of satyrization based on detection of interspecific sperm in spermathecae may underestimate the rates of this form of reproductive interference in nature.

Satyrization of *Ae. aegypti* by *Ae. albopictus* has been particularly studied since 2010. Interspecific mating has been detected in wild-caught females from four continents (Bargielowski et al., 2015a) and it has been shown that heterospecific male accessory gland extracts render *Ae. aegypti*, but not *Ae. albopictus*, females refractory to further mating (Tripet et al., 2011). The evolution of resistance to satyrization also has been documented, as interspecific mating is higher in *Ae. aegypti* females from populations allopatric to *Ae. albopictus*, than in females from sympatric populations (Bargielowski et al., 2013; Lounibos et al., 2016b). In my work I showed that *Ae. aegypti* females may be rendered refractory to further mating, even if *Ae. albopictus* sperm are not transferred to spermathecae. My results strengthen the understanding of reproductive interference as a mechanism to explain the rapid competitive displacements of *Ae. aegypti* by *Ae. albopictus* that occurred in the southeastern USA and Bermuda.
Asymmetric reproductive interference between \textit{Ae. albopictus} and two \textit{Aedes} species of the subgenus \textit{Stegomyia}, \textit{Ae. polynesiensis} and \textit{Ae. cretinus}, have been reported (Giatropoulos et al., 2015; Gubler, 1970a). Interspecific crosses between \textit{Ae. albopictus} and \textit{Ae. polynesiensis} or \textit{Ae. cretinus} generate no hybrids. In cage experiments, most of the \textit{Ae. polynesiensis} females were inseminated and rendered refractory to further mating after exposures to \textit{Ae. albopictus} males. Results of the reverse cross showed that \textit{Ae. polynesiensis} males do not inseminate \textit{Ae. albopictus} females (Gubler, 1970a). Gubler (1970b) believed that reproductive interference was the principal cause of the rapid elimination of \textit{Ae. polynesiensis} by \textit{Ae. albopictus} that occurs under laboratory conditions. However, a field trial that released \textit{Ae. albopictus} in a small atoll in the Pacific, failed to demonstrate displacement of indigenous \textit{Ae. polynesiensis} by \textit{Ae. albopictus} (Rosen et al., 1976).

Satyrization has been suggested as a mechanism to explain the spread of invasive \textit{Ae. albopictus} in Athens, Greece and the reduction in distribution of native \textit{Ae. cretinus}. Interspecific crosses between \textit{Ae. cretinus} and \textit{Ae. albopictus} showed that \textit{Ae. cretinus} females are more likely to engage in heterospecific mating (58%), than \textit{Ae. albopictus} females (1%) (Giatropoulos et al., 2015). The results of these authors on egg viability resemble the findings of my experiments described in chapter 3. Approximately 42\% of \textit{Ae. cretinus} females were not inseminated by \textit{Ae. albopictus} males, however only 17.5\% of the females previously exposed to heterospecifics, were able to mate with conspecifics and laid viable eggs. In my experiments, 46.7\% of the \textit{Ae. aegypti} females that were exposed to \textit{Ae. albopictus} did not have sperm in the spermathecae, and a mean of 16.3\% of the \textit{Ae. aegypti} females that were exposed to conspecifics,
after being confined with *Ae. albopictus* males, laid viable eggs. Results from both experiments suggest that many *Ae. aegypti* and *Ae. cretinus* females that do not have *Ae. albopictus* sperm in their spermathecae are refractory to further mating with conspecifics as evidenced by production of inviable eggs. However, in the case of *Ae. cretinus* more detailed studies are necessary to confirm if satyrization is occurring without heterospecific sperm transfer.

Reproductive interference could also be a cause of the abrupt decline that the native species *Ae. (Stegomyia) guamensis* suffered in Guam after the invasion of *Ae. albopictus* in 1944 (Lounibos, 2007). Rozeboom and Bridges (1972) suggested that the reduction of *Ae. guamensis* was caused by competition with *Ae. albopictus*, but the specific mechanisms were not studied.

Satyrization without insemination has been reported in other insects, such as the dermestid beetles *T. glabrum* and *T. inclusum*. In interspecific crosses between these beetle species, refractoriness to mating with conspecifics and oviposition of sterile eggs occurred when *T. glabrum* females copulated with *T. inclusum* males, but not after the reverse cross. *Trogoderma inclusum* males transfer spermatophores to the bursa of *T. glabrum* females, but sperm usually do not reach the spermathecae. It was suggested that transfer of male accessory gland material had occurred, despite the lack of sperm storage in the spermathecae (Vick, 1973). This example resembles the findings of my experiments, as postmating responses may occur even if heterospecific sperm are not stored in spermathecae. My results suggest that *Ae. albopictus* male accessory gland products and sperm may be transferred to the female bursa, but not to the
spermathecae, rendering *Ae. aegypti* females refractory to further mating even if they are not inseminated.

In my work I also developed an innovative technique using microscopy to evaluate the insemination status of live *Ae. aegypti* females, a procedure that had not been available previously. This technique preserves the female alive for subsequent experiments or observations. Using this technique I was able to demonstrate that satyrization of *Ae. aegypti* females by *Ae. albopictus* males occurred without evidence of insemination. The application of this technique was critical because it allowed me to check if a female’s spermathecae contained sperm after interspecific mating, and subsequently to expose each of the females without sperm to conspecifics. This technique also allowed me to study transfer of labelled semen from males to inseminated or uninseminated females.

This new technique was fundamental for the development of my research and it has several potential uses in future studies. It could be applied in experiments in which it is necessary to keep the live female intact in order to perform other procedures after detecting insemination status. This technique would be useful for the determination of insemination status in live females before performing behavioral tests, or in experiments in which mated females are required for subsequent observations, e.g. oviposition or vertical transmission of virus. An advantage of this technique is that it requires only basic supplies and equipment typically found in most entomology laboratories.

My work also illuminates mechanisms of sperm storage in mosquitoes. Previous observations have shown sperm movement after dissecting *Ae. aegypti* spermathecae in saline solution. However, it was unclear if motility was due to the effect of the saline
solution on sperm (Degner and Harrington, 2016). Although, I was not studying sperm motility, my in vivo observations of chapter 2 confirm that sperm remain active in spermathecae of live Ae. aegypti females (Degner and Harrington, 2016). I have only tried to detect insemination status in live Ae. aegypti females. If this technique is applicable to other flies, it would be interesting to study sperm motility in live individuals of other species, e.g. Anopheles gambiae. Observations of dissected spermathecae of this mosquito have shown that sperm in the single spermatheca start to be active only after they have been stored for more than 24 hours. In recently mated An. gambiae females, sperm are apparently inactive (Verhoek and Takken, 1994). Stronger conclusions could be made if observations were to be performed on live females.

The experiments of this dissertation on spermathecal usage also provide new information on the mating biology and sperm storage in Ae. aegypti and Ae. albopictus. Where the distributions of these two invasive species overlap, interspecific mating is facilitated as both Ae. aegypti and Ae. albopictus mate around hosts during the day, typically shortly after dawn (Gubler and Bhattacharya, 1972; Hartberg, 1971) and before dusk (Basio and Magluyan, 1975; Clements, 1999). However, differences in the reproductive biology of these two species were detected by De Jesus and Reiskind (2016) who showed that in intraspecific matings Ae. albopictus store more sperm in their spermathecae than Ae. aegypti, and an increase in fecundity occurs in Ae. albopictus females, but not in Ae. aegypti, that mate with larger males. My experiments determined that larger Ae. albopictus females have more spermathecae with sperm than smaller ones, after intraspecific and interspecific crosses. No influence of female
size was detected in *Ae. aegypti*, which indicates differences in the reproductive biology of these related species.

In conclusion my work describes an innovative technique using microscopy to detect presence or absence of sperm in spermathecae of live *Ae. aegypti* females without dissection, a procedure that had not been available previously. This technique was used to demonstrate that satyrization of *Ae. aegypti* females by *Ae. albopictus* males may occur even if heterospecific sperm are not transferred to spermathecae, suggesting that analyses of spermathecal contents may underestimate interspecific matings. Differences of the effect of female size on spermathecal usage in *Ae. aegypti* and *Ae. albopictus* imply differences in their mating biology. My results enlighten our understanding of satyrization as a mechanism to explain competitive displacements of *Ae. aegypti* by *Ae. albopictus*, and provide important knowledge on their mating biology.
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BIOGRAPHICAL SKETCH

María Cristina was born and raised in Bogotá, Colombia. From a young age she was exposed to the world of entomology as her mother worked for several decades as a medical entomologist. Her stories, anecdotes and the many times she went with her on field trips and to the laboratory during her childhood and youth, strongly marked her interest towards the amazing world of insects and biology. Furthermore, growing up in Colombia, a tropical country where vector-borne diseases represent a serious public health problem, made her interest grow even more towards medical entomology. She studied biology at Universidad de los Andes, Colombia. After she finished her bachelor’s degree, she worked as a young researcher at the Entomology Laboratory, Instituto Nacional de Salud (INS), Colombia, on a project to evaluate impregnated bed nets to control vectors of *Leishmania*. She had opportunities to visit communities affected by this disease and to witness firsthand its social and economic burdens. She pursued a master’s degree at Universidad de los Andes, and she became involved in a project focused on the ecology and control of biting midges. She performed her thesis in communities affected by extremely high biting rates of midges. Her fascination for medical entomology and her aspiration for being a very well prepared researcher, made her pursue doctoral studies in medical entomology at the Florida Medical Entomology Laboratory at the University of Florida, with Dr. Phil Lounibos as her advisor and Dr. Jorge Rey as her co-advisor. Her dissertation focused on reproductive interference and spermathecal use in invasive mosquito vectors.