THE TIMING OF REPRODUCTIVE EVENTS IN THE MOSQUITO *AEDES AEGYPTI*

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THE TIMING OF REPRODUCTIVE EVENTS IN THE MOSQUITO *AEDES AEGYPTI*

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The mosquito *Aedes aegypti* is the primary vector of dengue, yellow fever, chikungunya, and Zika viruses. Despite decades of effort to curb transmission of these diseases, this mosquito's continued status as the most proficient arbovirus vector highlights the need for new and improved vector control methods. Manipulating entire mosquito populations in the wild is a promising avenue by which mosquitoes may be controlled. Typically, such strategies aim either to suppress a wild population by limiting reproduction or to replace a population with mosquitoes with a desirable trait (such as refractoriness to disease). Regardless of the type of manipulation, these strategies require that altered individuals (usually reared in laboratories or factories) are deployed into the wild to compete with and mate with wild individuals.

While mosquito reproduction has been studied for decades, many questions still remain to be answered that could help to improve mosquito release strategies. The purpose of this dissertation was to answer fundamental questions surrounding *Ae. aegypti* reproduction that will contribute to the development of new or enhanced mosquito control strategies. Specifically, it addresses the timeline with which several reproductive events occur and lays a foundation for future investigations to describe the cellular and molecular underpinnings of important reproductive events.

First, I addressed the question of when *Ae. aegypti* females mate more than once. While consensus in the literature suggested that they do occasionally mate more than once, some studies suggest that re-mating occurs soon after a female's first mating, and others claim that females only mate after they have undergone several gonotrophic cycles. Using males with fluorescently labeled sperm, I demonstrate that females are most likely to mate a second time within 2 h of their first mating, that almost no re-insemination occurs after 24 h post-mating (hpm), and that no females re-mate after they have begun laying eggs.

Given this 24 hpm period in which behavior drastically shifts, I wondered if other aspects of reproductive physiology also follow similar timelines. Studies of other mosquitoes' sperm suggested that sperm are initially equipped with a thick glycocalyx, but that this outermost layer is removed some time after mating. To understand whether this occurs in *Ae. aegypti*, when it occurs, and if it is typical of all sperm, I used cryo-electron microscopy to image sperm from the male prior to insemination and at different times post-insemination from the female. Within 24 hpm, sperm indeed shed a thick outer glycocalyx. Motility assays over the same 24 h period indicated that within 8 h, sperm had achieved their maximum motility. Finally, I tested whether oviposition is initiated and fertility is achieved during this period. Both reached their peak at ~16 hpm, with fertility gradually setting in beginning at 4 hpm and oviposition experiencing a sharp increase after 12 hpm.

After investigating *Ae. aegypti* reproduction in the laboratory, I aimed to describe aspects of their reproductive biology in an epidemiologically relevant field setting. I intensively sampled *Ae. aegypti* in Medellín, Colombia, by aspirating resting

adults from homes. By recording data on each female's mating status, parity status, and whether she had taken a blood meal and developed eggs, I compared virgin and mated females' behavior and physiology. Females frequently took blood meals prior to mating. However, blood fed virgins' abdominal distension was significantly lower than that of mated females, suggesting that virgins may take smaller blood meals. Some virgins also produced eggs, and those that did produced a number of eggs similar to their mated counterparts. These observations provide the first comprehensive comparison of mated and virgin *Ae. aegypti* feeding behavior and reproductive physiology in a field setting.

Knowing when critical reproductive events occur is a critical first step to understanding how they occur. To enable future investigations of the cellular and molecular mechanisms that govern reproductive events, I developed a comprehensive proteome for both *Ae. aegypti* sperm and seminal fluid. Building on previous work by Sirot et al. (2011) and using state-of-the-art mass spectrometry technology, I was able to identify over 870 putative sperm proteins and 280 putative seminal fluid proteins. These findings were largely corroborated and supported by transcriptomes that I generated from the testes and male accessory glands. The findings in this dissertation will support future investigations of crucial reproductive processes that may be manipulated for the purposes of vector control.

BIOGRAPHICAL SKETCH

Ethan grew up in Saint Peter, MN, where he enjoyed his role as the youngest of six children and the son of a Lutheran minister and an elementary school teacher. From early on, he was fascinated with the natural world, and in particular all things creepy-crawly—snakes, frogs, and especially insects. He loves and cherishes his frequent trips to the local fishing pond, yearly camping trips in the Boundary Waters of northern Minnesota, and annual family vacations to national parks around the United States. He attended Minnesota Valley Lutheran High School, where he was involved in many extra-curricular activities, including football, baseball, jazz band, math team, and knowledge bowl. During this time he also developed a passion for fishing, and decided to study biology in college with the hope of pursuing a career in natural resources.

Ethan attended Gustavus Adolphus College in his hometown, where his passion for biology grew. Dr. Margaret Bloch-Qazi was gracious enough to teach him "fly-pushing" in her *Drosophila* lab, and Dr. Joel Carlin mentored him through a senior honor's thesis studying an estuarine minnow. During the summers, he worked internships with the Minnesota Department of Natural Resources conducting fisheries surveys, and with the US EPA in Gulf Breeze, FL, developing toxicity tests for estuarine fishes. Upon graduation, he took an internship in Panamá with the Smithsonian Tropical Research Institute, during which he studied leaf-cutter ant foraging behavior. There, he re-discovered his love of insects, both in his work and via countless treks into the tropical rainforest at night. Wanting to study insects that directly impact human lives, he had an epiphany during one of his father's sermons in which he decided to pursue medical entomology. A quick Google search led him to his dissertation adviser, Laura Harrington at Cornell University.

At Cornell, Ethan developed a passion for mosquitoes and for undergraduate mentorship and teaching. He also continued a passion for enjoying the great outdoors, trolling for salmon and lake trout in his canoe on Cayuga Lake, bowhunting, winning pub trivia with the Secret Canadians, and karaoke. His membership in the Graduate Christian Fellowship and Cross of Christ Lutheran Church provided him with friends that will last him a lifetime. Upon finishing his PhD, Ethan will begin a faculty position as an Assistant Professor of Biology at Wisconsin Lutheran College in Milwaukee, WI. While he is excited to be close to family, Cornell, Ithaca, and the friendships he established during his graduate years will always be near and dear to his heart.

I dedicate this dissertation to:

My Lord and Savior, Jesus Christ, together with the Father and Holy Spirit. Without Him nothing is possible, and I would be nothing. Every good and perfect gift comes from above. All glory and praise to Him.

My parents, Rev. Charles and Linda Degner, for encouraging me to pursue my passions from early on, reading to me, quizzing me on math problems, taking me fishing, the opportunities to see and fall in love with the natural world, investing both time and money in my education, never-ending support, leading by example with a service-above-self attitude, and unconditional, unbounded love. I would not be the person I am today without these wonderful human beings.

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Olivia Winokur, Lindsay Baxter, Kevin Pritts, Julie Geyer, Julian Montijo, and all of the undergraduates of the Harrington lab. I am grateful for the good times we have had, the help you have provided, the brainstorming sessions, and the friendship we have shared.

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My collaborators and co-authors: Jade Noble, Lena Kourkoutis, Adam Hatala, Yasir Ahmed-Braimah, Kirill Borziak, Steve Dorus. How fun it has been to combine our expertise to push back the frontiers of science.

The entomology administrative staff and Comstock support staff: Cheryl Gombas, Stephanie Westmiller, Lisa Marsh, Lisa Westcott, Amy Arsenault, Rita Stuckey, Ken Ayers, and our faithful custodians. Thank you for all of your help with paperwork, spills, maintenance. You are unsung heroes.

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LIST OF ABBREVIATIONS

µg ..... microgram
µL ..... microliter
µm ..... micrometer
Ac ..... acrosome
Acp36DE ..... SFP in *Drosophila melanogaster*
APEX ..... absolute protein expression
ATP ..... adenosine triphosphate
Ax ..... axoneme
BLAST ..... basic local alignment search tool
bp ..... base pair
BRH ..... best reciprocal hits
$CaCl_2$ ..... calcium chloride
cDNA ..... complementary deoxyribonucleic acid
CID ..... collision-induced dissociation
CRISPR ..... clustered regularly interspaced palindromic repeats
d ..... day
Da ..... Dalton
DAPI ..... 4′,6-diamidino-2-phenylindole
DDT ..... dichlorodiphenyltrichloroethane
DENV ..... dengue virus
DI ..... deionized
dpe ..... days post-eclosion
DsRed ..... red fluorescent protein
ECD ..... Ethan Caleb Degner
FDR ..... false discovery rate
FW ..... femur width
FT ..... Fourier transform
fwhm ..... full width at half maximum
G ..... glycocalyx
GCaMP3 ..... fluorescent calcium sensor in *Drosophila melanogaster*
GLM ..... generalized linear model
GO ..... gene ontology
h ..... hour
hpm ..... hours post-mating
i.d. ..... inner diameter
IRB ..... Institutional Review Board
ISD ..... inter-sclerite distance
IT ..... ion trap
KCl ..... potassium chloride
KEGG ..... Kyoto Encyclopedia of Genes and Genomes
KH_2PO_4 ..... monopotassium phosphate
kV ..... kilovolt
L L
M ..... molar

m/z ..... mass to charge ratio
MAG ..... male accessory gland
MAPK ..... mitogen-activated protein kinase
Mb ..... megabase pair
MD ..... mitochondrial derivative
min ..... min
miRNA ..... micro-ribonucleic acid
mL ..... milliliter
mm ..... millimeter
mM ..... millimolar
mRNA ..... messenger ribonucleic acid
MS ..... mass spectrometry
ms ..... millisecond
MS/MS ..... tandem mass spectrometry
mzML ..... file format for MS/MS data
N ..... nucleus
Na_2HPO_4 ..... disodium phosphate
NaCl ..... sodium chloride
nanoLC-ESI-MS/MS ..... nano-liquid chromatography-electrospray ionization-tandem mass spectrometry
nm ..... nanometer
Octβ2R ..... *Drosophila melanogaster* octopamine receptor
Orco ..... odorant receptor coreceptor
PBS ..... phosphate buffered saline
PCR ..... polymerase chain reaction
PEBII ..... SFP in *Drosophila melanogaster*
PEBme ..... SFP in *Drosophila melanogaster*
Pkd2 ..... calcium channel in *Drosophila melanogaster*
ppm ..... parts per million
protXML ..... file format for protein identifications derived from MS/MS data
PSM ..... peptide spectral match
RAW ..... file format for MS/MS data
RNA ..... ribonucleic acid
RNA-seq ..... ribonucleic acid sequencing
s ..... second
SD ..... standard deviation
SDS-PAGE ..... sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE ..... standard error
SFP ..... seminal fluid protein
S-LAP ..... sperm leucyl-aminopeptidase
TCA ..... tricarboxylic acid
TPM ..... transcripts per million
x *g* ..... acceleration due to gravity, or relative centrifugal force
y ..... year

CHAPTER 1

INTRODUCTION

Aedes aegypti is a global threat to human health and life. As the primary vector of dengue, yellow fever, chikungunya, and Zika viruses, it is responsible for severe morbidity, mortality[1], and substantial economic strain on public health infrastructure[2]. Efforts to limit and eliminate it began shortly after the discovery that *Ae. aegypti* transmits yellow fever in 1900[3]. By the 1960s, following widespread campaigns using DDT, 18 New World nations had achieved eradication. However, due to social, political, and biological factors, *Ae. aegypti* has re-invaded every country that had once eliminated it[3]. At present, most mosquito control efforts are insecticide-based, but their long term success at reducing dengue burdens is dubious[4]. Public health awareness campaigns have been successful at limiting mosquito densities by encouraging the removal of possible breeding sites[5], but are unlikely to result in eradication. Thus, despite these efforts, *Aedes aegypti* is still the most prolific vector of arbovirus diseases[1]. New and improved efforts to control *Aedes aegypti* are clearly needed.

A multitude of new and innovative methods for controlling *Ae. aegypti* are under development or currently being tested. For example, efforts to drive an intracellular bacterium, *Wolbachia*, into wild populations of *Ae. aegypti* are underway in several countries[6]. This intracellular symbiont confers dengue resistance to *Aedes aegypti*[7], and it also induces a reproductive phenotype that (theoretically) allows it to

spread and fix in a population[8]. Attempts to suppress populations using inundative releases of males are also being tested in several locations and have achieved some success; genetically modified males whose offspring cannot reach adulthood have been deployed to cause population reductions of 90% or more[9]. Finally, manipulation of mosquito development in the field may be accomplished by new insecticides that avoid traditional modes of action and are target specific. One example of these is a larvicide that consists of transgenic yeast, that, when ingested, knocks down critical developmental genes using the mosquito larvae's RNAi pathway[10].

All of the above approaches to mosquito control stem from decades of investigations into the basic biology of *Ae. aegypti*. However, the current knowledge base of *Ae. aegypti* reproduction remains incomplete. It has long been known that female behavior and physiology drastically change after mating[11, 12, 13, 14, 15, 16, 17, 18], but two crucial gaps exist in our understanding of this process: the timing with which female behavior and physiology changes, and the events that occur within her reproductive tract that enable her to fertilize and lay eggs. Herein, I have provided detailed chronologies of several reproductive events, including polyandry (that is, multiple mating by females), sperm modification within the female reproductive tract, oviposition behavior, and the onset of female fertility. In order to better understand the molecular drivers of reproduction, I have also catalogued over 1000 sperm and seminal fluid proteins—a dataset that will serve as the basis for future investigations of molecular pathways that govern *Ae. aegypti* reproduction. Finally, I have described *Ae. aegypti* feeding and mating behavior in Medellín, Colombia—a location in which inundative releases of *Wolbachia*-infected mosquitoes are being tested. Ultimately,

this work provides an understanding of *Ae. aegypti* behavior and physiology that will improve existing mosquito control strategies and may lead to new targets by which reproduction could be manipulated in a field setting.

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CHAPTER 2

A MOSQUITO SPERM'S JOURNEY FROM MALE EJACULATE TO EGG: MECHANISMS, MOLECULES, AND METHODS FOR EXPLORATION[*]

Summary

The fate of mosquito sperm in the female reproductive tract has been addressed sporadically and incompletely, resulting in significant gaps in our understanding of sperm-female interactions that ultimately lead to fertilization. As with other Diptera, mosquito sperm have a complex journey to their ultimate destination, the egg. After copulation, sperm spend a short time at the site of insemination where they are hyperactivated and quickly congregate near the entrance of the spermathecal ducts. Within minutes, they travel up the narrow ducts to the spermathecae, likely through the combined efforts of female transport and sperm locomotion. The female nourishes sperm and maintains them in these permanent storage organs for her entire life. When she is ready, the female coordinates the release of sperm with ovulation, and the descending egg is fertilized. Although this process has been well studied via microscopy, many questions remain regarding the molecular processes that coordinate sperm motility, movement through the reproductive tract, maintenance, and usage. In this review, we describe the current understanding of a mosquito sperm's journey to the egg, highlighting gaps in our knowledge of mosquito reproductive biology. Where

[*] Presented with permission from John Wiley & Sons, Inc., from the original published article: Degner, E. C., L. C. Harrington. (2016). A mosquito sperm's journey from male ejaculate to egg: mechanisms, molecules, and methods for exploration. Mol. Reprod. Dev. 83(10):897-911.

insufficient information is available in mosquitoes, we describe analogous processes in other organisms, such as *Drosophila melanogaster*, as a basis for comparison, and we suggest future areas of research that will illuminate how sperm successfully traverse the female reproductive tract. Such studies may yield molecular targets that could be manipulated to control populations of vector species.

Introduction

The battle to control mosquito disease vectors is among the greatest public health challenges of our time. *Anopheles* mosquitoes cause nearly 200 million cases of malaria annually, and kill approximately 600,000 people each year (WHO 2014). *Aedes aegypti* and *Ae. albopictus* transmit dengue viruses, which cause more than 100 million infections (Bhatt et al. 2013), as well as emerging viral threats such as chikungunya (Staples and Fischer 2014) and Zika (Fauci and Morens 2016). Many mosquito-borne pathogens have no commercially licensed vaccine or cure; even for those with a cure, such as malaria, drug resistance poses a serious challenge (Murai et al. 2015). The most effective tools against mosquito-borne pathogens therefore remain those focused on regulating vector populations

While most mosquito control strategies focus on killing adults or larvae, strategies that target mosquito reproduction hold significant promise. To date, studies of reproduction have focused primarily on female biology and aspects of egg development and deposition, but relatively little work has been conducted on male contributions to reproduction. A deeper understanding of sperm motility and

interactions with the female reproductive tract will be valuable to devising field-ready, practical targets for use in new control strategies.

Studies of mosquito sperm may also advance our understanding of sperm biology in other animals, including humans. Many aspects of sperm biology are conserved across Animalia. *Drosophila melanogaster* is the model organism of choice for many biological phenomena, but the unusual length of their sperm (1.9 mm) makes some aspects of their biology – such as sperm motility – difficult to study. Mosquitoes are an excellent surrogate model, as their sperm are about one-eighth the length (depending on species) and much more tractable for investigation.

Here, we describe the journey of mosquito sperm through the female reproductive tract to its ultimate destination, the egg. We first describe modulators of sperm motility, as these are critical to understanding how sperm move through the reproductive tract. A sperm's course throughout the female mosquito has been well characterized via microscopy, but little attention has been paid to molecular interactions among sperm, seminal fluid, and the environment within the female that assist sperm on their voyage. Here, we assemble what is known of mosquito sperm and highlight future avenues of research, paying special attention to areas where molecular mechanisms are likely critical to sperm viability and male reproductive success. Much of our knowledge in mosquitoes comes from the important vector *Ae. aegypti*, thus we focus on this species as a model but include other mosquito species for which information is available. We also glean insight from *Drosophila melanogaster*, other insects, or other organisms whose biology has been studied in greater detail for phenomena that have not been adequately evaluated in mosquitoes.

Reproductive tract morphology

A description of the arena that sperm traverse is necessary in order to understand their journey through the female reproductive tract (Figures 2.1 and 2.2). Males transfer sperm and semen directly into a sac-like organ inside the female gonotreme, or vagina (Giglioli 1963; Spielman 1964). This organ acts as a temporary holding site for sperm. After a ~40-sec delay (Spielman 1964; Jones and Wheeler 1965a), sperm begin to travel from this organ up narrow ducts into one to three spermathecae. The spermathecae are chitinous, spherical reservoirs in which sperm are stored long-term. Sperm are maintained in these capsules for a female's entire life, nourished by glandular cells adjoining the spermathecae (Clements and Potter 1967; Pascini et al. 2012; Pascini et al. 2013). Ultimately, sperm travel back down these same ducts and fertilize eggs that pass down the common oviduct and out the gonotreme (Figure 2.3).

Two mosquito subfamilies are currently recognized within Culicidae: Culicinae and Anophelinae (Mitchell et al. 2002; Harbach 2007). Members of each subfamily possess slightly different reproductive tract morphology. In Culicinae, the sac-like organ into which sperm are deposited is called a bursa (also referred to as a bursa copulatrix or bursa inseminalis), a reservoir that is adjacent to and attaches at the base of the common oviduct (Figure 2.1). In Anophelinae, the organ with the same function is termed the atrium, and is simply an expanded region of the common oviduct that accommodates the ejaculate when initially transferred. In contrast to Culicinae, a mating plug quickly forms in the atrium of most Anophelinae via a combination of the male ejaculate and female secretions (Giglioli and Mason 1966;

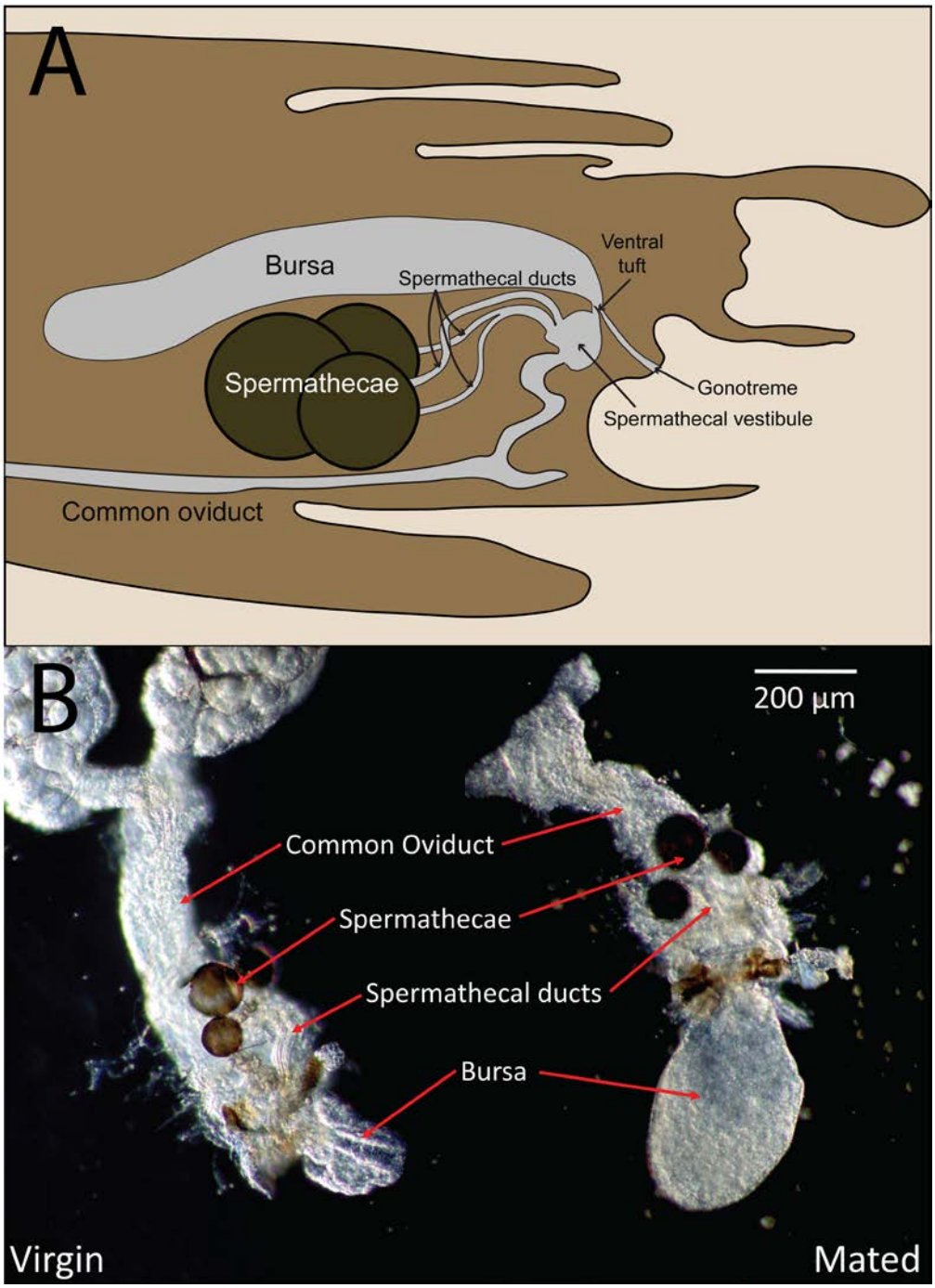

Figure 2.1. Typical reproductive tract of female Culicinae mosquitoes. **A**: Simplified sagittal diagram with relevant anatomy labeled. For detailed anatomical drawing of the final abdominal segments, see Figure 2.2. **B**: Dissected reproductive tract of virgin (left) and mated (right) *Aedes aegypti* females. The bursa is inflated by semen in the mated female. Scale bar, 200 µm.

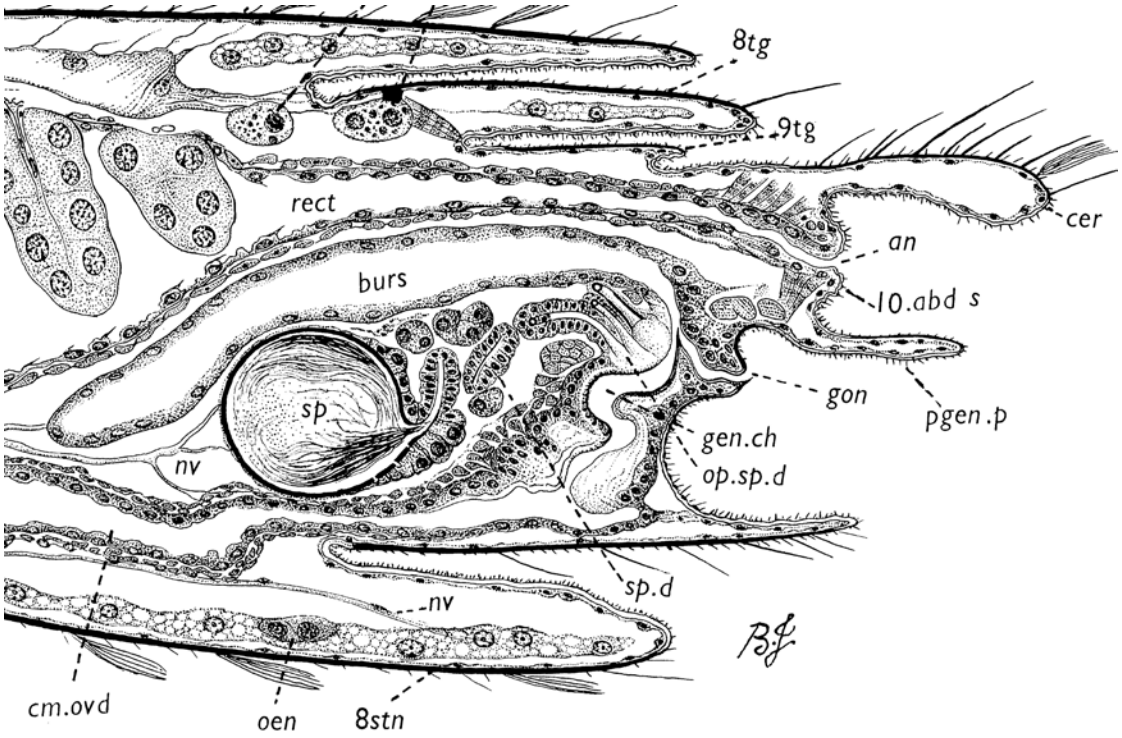

Figure 2.2. Detailed anatomical drawing from which Figure 2.1A is derived (Jobling and Lewis 1987). Used with permission from the Wellcome Trust (London, UK).

Mitchell et al. 2015); a noted exception is the New World species *An. albimanus*, in which a mating plug does not form after copulation (Mitchell et al. 2015). The number of spermathecae varies by subfamily as well: Anophelinae has only one, whereas most (but not all) Culicinae have three (Edwards 1941; Yuval 2006).

Sperm morphology

Mosquito sperm are long and slender. Unlike mammalian sperm, mosquito sperm heads are as wide as the tail, with a diameter of 0.5–0.6 µm (Clements and Potter 1967; Tongu 1968). The head contains the nucleus and is identifiable by its rigidity, in contrast to the undulating flagellum (Figure 2.4). The flagellum consists of

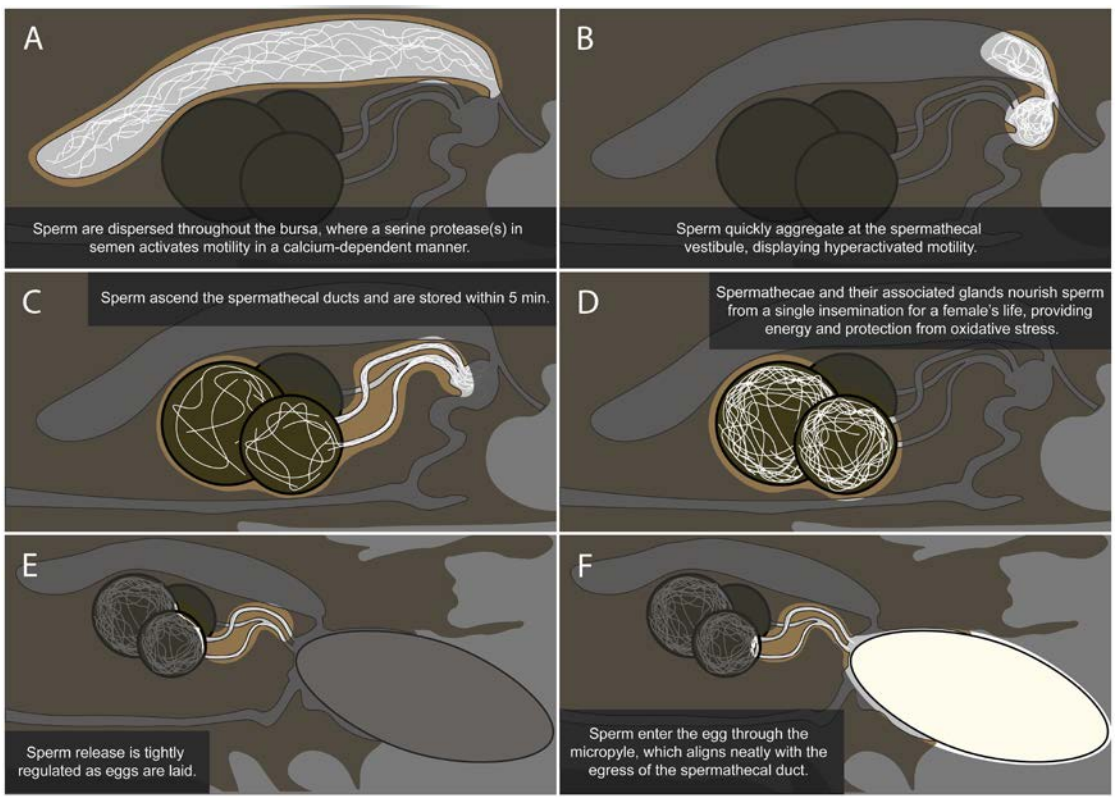

Figure 2.3. Frame-by-frame model of sperm path through Culicinae female reproductive tract from insemination (**A**) to fertilization (**F**).

two mitochondrial derivatives, which extend the length of most of the flagellum, and an axoneme, a microtubular structure responsible for motility (Clements and Potter 1967; Bao et al. 1992). Mosquito sperm axonemes have 19 microtubules arranged in two concentric circles of nine around a lone central tubule (9 + 9 + 1) (Breland et al. 1966; Clements and Potter 1967; Swan 1981) – an arrangement that contrasts with most other insect axonemes studied to date, which have a pair of central tubules (9 + 9 + 2) (Phillips 1969). The functional significance of this deviation in mosquitoes is unknown. Average mosquito sperm length varies by species, from 100 µm in *An. albimanus* (Klowden and Chambers 2004) to 570 µm in *Culiseta inornata* (Breland et al. 1968). Klowden and Chambers (2004) found that average sperm length across six

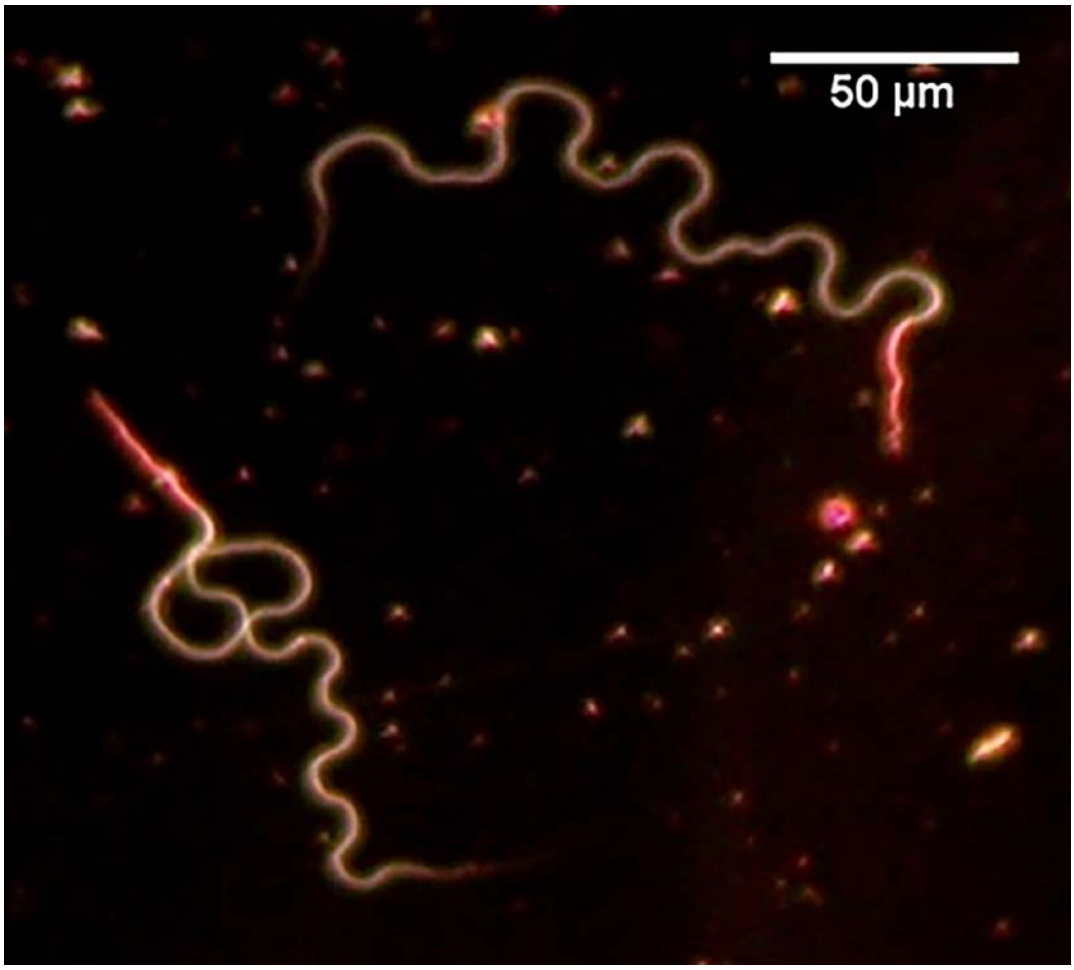

Figure 2.4. *Aedes aegypti* sperm. Rigid heads are stained with ethidium bromide. Scale bar, 50 µm. Used with permission from Alongkot Ponlawat.

mosquito species correlated with spermathecal volume, implying that these measurements may have sexually coevolved. Experimental selection of *D. melanogaster* sperm length and seminal receptacle size yielded similar insights (Miller and Pitnick 2002).

Klowden and Chambers (2004) also identified significant variation in sperm length within *An. gambiae*, *An. quadriannulatus*, and *An. darlingi*, whereas sperm size was uniform in other species, such as *An. freeborni* and *Ae. aegypti*. In one study with

An. gambiae, average sperm length varied with body size (Voordouw et al. 2008). While these few studies have identified intriguing variation in sperm length for some species, the contribution of different length sperm to fertilization remains unknown. In contrast to Lepidoptera, which produce a class of short sperm without nuclei (Friedlander and Gitay 1972; reviewed in Friedlander 1997), all mosquito sperm contain nuclear DNA, suggesting that even short sperm are capable of fertilization (Klowden and Chambers 2004). Some *Drosophila* species produce both long and short nucleated sperm, but only long sperm fertilize eggs (Snook et al. 1994; Snook and Karr 1998). Several hypotheses have been proposed in other insects to explain the adaptive significance of having a class of sperm that does not contribute to fertilization, including nutritional provisioning by extra sperm, facilitating sperm transport, or preventing the receipt or storage of another male's sperm (reviewed in Swallow and Wilkinson 2002). If and how polymorphic sperm may be adaptive in mosquitoes is an intriguing and underexplored topic of research.

Sperm motility

Thaler et al. (2013) provide the most comprehensive description of in vitro mosquito sperm motility to date using an elegant series of experiments. They described three discrete flagellar wave patterns in *Culex quinquefasciatus* sperm: a long-wavelength, low-amplitude flagellar wave (type A); a double-wave consisting of a short-wavelength, low-amplitude wave superimposed over a long-wavelength, high-amplitude wave (type B); and a rapid, helical wave (type C). Such a range of motility is consistent with that observed in the sperm of *Ae. aegypti* (Jones and Wheeler

1965b), and is similar to the waveform diversity described in *D. melanogaster* (Yang and Lu 2011). Thaler et al. (2013) further found that *Cx. quinquefasciatus* sperm are capable of swimming both forward and backward. Bidirectional locomotion has been described in several other Dipterans, including the hump-backed fly, *Megaselia scalaris* (Curtis and Benner 1991); three flies in the family Tephritidae – *Ceratitis capitata, Bactrocera dorsalis*, *B. oleae* (Baccetti et al. 1989); and *D. melanogaster* (Kottgen et al. 2011). The significance of backwards swimming by mosquito sperm has not been examined, whereas it likely helps orient sperm correctly for fertilization in *D. melanogaster* (Kottgen et al. 2011).

Thaler et al. (2013) also revealed molecular regulators of the different waveforms and direction of *Cx. quinquefasciatus* sperm motility. Sperm are immotile in the male's seminal vesicle prior to ejaculation, and sperm dissected from the seminal vesicle remain weakly motile. Upon mixing with semen from the accessory glands, however, sperm progress sequentially from A- to B- to C-type motility. This progression is calcium-dependent; in its absence, very few sperm are motile, and those that are motile exhibit backwards (tail-leading) type-A motility. In the absence of accessory glands, complete progression to type-C motility could be induced by the addition of trypsin, suggesting a similar serine protease is present in semen that may be critical for full sperm activation. Transition from type-B to type-C motility is mediated by protease-dependent activation of the mitogen-activated protein kinase (MAPK) phosphorylation pathway ; sperm incubated with MAPK inhibitors are incapable of type-C motility. A similar process of activation has also been described in the water strider, *Aquarius remigis*, in which trypsin activates the MAPK pathway via

the protease activated receptor PAR2 (Miyata et al. 2012). Protease-dependent activation of sperm has also been described in other distantly related insect orders, including Lepidoptera (Shepherd 1974; Aigaki et al. 1987; Osanai and Baccetti 1993; Aigaki et al. 1994) and Orthoptera (Osanai and Baccetti 1993), suggesting a highly conserved mechanism of sperm activation across Insecta.

Intracellular calcium concentration is a highly conserved regulator of sperm motility in animals (Suarez et al. 1993; Bannai et al. 2000; Gao et al. 2003; reviewed in Darszon et al. 2006). In insects, specific proteins that control sperm calcium concentrations are best described in *D. melanogaster*. The calcium channel Pkd2 is localized at sperm heads and the tips of sperm tails, and is required for successful sperm storage in the *D. melanogaster* seminal receptacle (Gao et al. 2003; Watnick et al. 2003). Pkd2 also plays a role in hyperactivation of sperm (Kottgen et al. 2011). The gene *CG34110* produces a protein predicted to be an upstream signaler of Pkd2 (Yang et al. 2011), as mutations of either *CG34110* or *Pkd2* result in similar sperm dysfunctions. Similar calcium channels have yet to be identified in mosquitoes, although an odorant-gated ion channel, Orco, localizes to the sperm flagella and may play a similar role in the regulation of intracellular osmolarity and motility in the mosquitoes *Ae. aegypti* and *An. gambiae* (Pask et al. 2011; Pitts et al. 2014). Orco forms a complex with an accompanying odorant receptor (Sato et al. 2008) and has been shown to activate mosquito sperm in the presence of various ligands (Pitts et al. 2014).

The importance of calcium channels in sperm movement raises the question of whether or not females can control fluxes of extracellular calcium or other ions

throughout their reproductive tract to modulate sperm movement – a possibility that should be considered in mosquitoes. Kaneuchi et al. (2015) recently used a transgenic fly expressing a fluorescent calcium sensor (GCaMP3) to image calcium fluxes in vivo in *D. melanogaster* oocytes; adapting their method to mosquitoes could elucidate the osmotic environment that sperm encounter in females.

Substrate viscosity may also modulate sperm motility. Curtis and Benner (1991) found that sperm velocity of the humpbacked fly, *Megaselia scalaris*, tripled when substrate viscosity was increased with 1% methylcellulose, and sperm converted to a more linear swimming form. Higher viscosity elicits a different waveform in sperm of the tunicate *Ciona intestinalis*, whose flagellar movement converts from a planar wave to a helical wave (Brokaw 1966). It is unclear how discrete waveforms may be induced by substrate viscosity, although Werner and Simmons (2008) hypothesized that increased mechanical stress on the sperm tail is converted into a biochemical signal (such as an influx of ions) by membrane proteins (Watson 1991).

Physical interactions with the reproductive tract itself may also be critical for sperm movement. Werner et al. (2007) and Werner and Simmons (2008) discussed the possibility that helical waveforms may maximize contact with narrow spermathecal ducts to assist sperm locomotion, allowing sperm to propel themselves by pushing against the duct rather than the fluid in the lumen of the reproductive tract. Nosrati et al. (2015) report that mammalian sperm may change their flagellar motion from a free-swimming, helical wave to a two-dimensional "slither" when within 1 µm of a solid surface. Similar interactions are easy to imagine in mosquitoes: In *Ae. aegypti*, for example, 250 µm-long sperm travel up 1.2–3.5 µm-wide ducts into spermathecae that

are half as wide as a sperm's total length (Jones and Wheeler 1965b; Clements and Potter 1967; Linley and Simmons 1981). Such crowded conditions lead to intimate contact with the reproductive tract and may facilitate locomotion.

Sperm aggregation at the spermathecal vestibule

Most female mosquitoes couple with males in flight and copulate shortly thereafter, during which time insemination occurs. *Ae. aegypti* males transfer seminal fluid and sperm into the bursa. Spielman (1964) tracked the movement of sperm in freshly inseminated females by flash-freezing females after insemination and observing sperm location in these fixed specimens by light microscopy. He noted that sperm are initially dispersed throughout the bursa but quickly aggregate at and orient towards the spermathecal vestibule (Figure 2.3), a small invagination of the reproductive tract near the entrance to the bursa and leading to the spermathecal ducts (Figure 2.1). Jones and Wheeler (1965a) noted that sperm displayed vigorous activity concentrated around the spermathecal vestibule. Our observations in *Ae. aegypti* support these accounts of strong, directed localization to the spermathecal vestibule with robust locomotion (Video 1[*]). These results are consistent with studies that report hyperactivation in *D. melanogaster* (Kottgen et al. 2011). Of the waveforms described by Thaler et al. (2013), it is unknown which are used in guiding sperm to the spermathecal vestibule, and it is possible that sperm switch from one form to another within the bursa. Due to the mass movement of sperm in the confined spaces of the bursa, we have thus far been unable to identify discrete waveforms of individual

[*] Video 1 can be accessed at URL: https://onlinelibrary.wiley.com/doi/full/10.1002/mrd.22653

sperm in our observations. To our knowledge, detailed descriptions of sperm activity in the atrium of Anophelinae do not exist, although such studies would further our understanding of sperm in this medically important subfamily.

Getting sperm to the spermathecal vestibule may be accomplished by chemotaxis, consistent with the results of Pitts et al. (2014), which demonstrated chemical activation of sperm in response to different ligands. Possible origins of the stimulus responsible for localization to the vestibule include the female accessory gland, the spermathecal duct glands, or the spermathecal glands (Clements and Potter 1967; Pascini et al. 2012; Pascini et al. 2013). Yet Jones and Wheeler (1965a) discounted all three possibilities, as they did not observe any in vitro activation of sperm in response to these organs – although these observations should be verified in vivo to truly understand sperm activation. Schnakenberg et al. (2011) showed that products from *D. melanogaster* spermathecal secretory cells contribute to the motility of sperm in a separate storage organ, the seminal receptacle. They eliminated these cells by using the promoter of genes specific to the spermathecal secretory cells to drive expression of a protein that induces apoptosis. With this tissue disabled, sperm in the seminal receptacle lost motility. Thus, secretion from one tissue of the reproductive tract can modulate sperm motility in a different organ. If specific sperm-activating molecules in the female mosquito reproductive tract are discovered, knowing where and when they are expressed will be important for understanding how they affect sperm. Heifetz et al. (2014) developed antibody-based methods to map the expression of several neurohormones throughout the *D. melanogaster* reproductive

tract over several hours; their methods may be adapted to identify reproductive molecules in mosquitoes.

Coagulation of semen has been reported for mosquitoes such as *Ae. albopictus* (Oliva et al. 2013), and a mating plug forms in many Anophelinae species (Giglioli and Mason 1966; Mitchell et al. 2015). Such changes in the seminal mass may create a viscous environment that influences sperm motility, as has been demonstrated in other organisms (Brokaw 1966; Curtis and Benner 1991). If confirmed, such a mechanism of sperm activation would complement a study by Rogers et al. (2009) that showed the importance of a key seminal fluid protein in *An. gambiae*. They demonstrated that the mating plug ensures successful sperm storage, and sperm storage was severely inhibited without the plug-forming enzyme transglutaminase. Similarly, the seminal fluid protein PEBme is required for effective mating plug coagulation and sperm storage in *D. melanogaster* (Avila et al. 2015).

Mechanisms of spermathecal filling

Sperm are rapidly stored in the spermathecae. *Ae. aegypti* sperm begin to enter the spermathecae as soon as 30 sec after mating, and spermathecal filling is completed in 300 sec (Spielman 1964; Jones and Wheeler 1965b). In most Culicinae, sperm typically only fill one of the lateral spermathecae and the median spermatheca (Jones and Wheeler 1965b; Oliva et al. 2013; personal observation). How the spermathecae are filled with sperm remains unclear, although sperm translocation to the spermathecae is broadly attributed to either sperm locomotion or active transport by the female reproductive tract – which are not mutually exclusive. To our knowledge,

the process of spermathecal filling in mosquitoes has only been examined in detail for *Ae. aegypti*. Jones and Wheeler (1965a; 1965b) performed several experiments in an attempt to elucidate how sperm reach the spermathecae. They noted that sperm moved rapidly in the bursa, and thus concluded that sperm locomotion alone is sufficient to fill the spermathecae. Yet, they also found that dead females did not store sperm, even if the bursa was inseminated. Therefore, some cooperation from the female was evident (Jones and Wheeler 1965a). Spielman (1964) suggests that the ventral tuft, a valve-like projection of the female reproductive tract ending in delicate hairs, may act as a gate to the spermathecal vestibule, preventing storage of a male's sperm until a female opens this valve (Figure 2.5). The stimulus that causes a female to grant this access is unknown, but this checkpoint may serve as a guard against interspecific matings (Carrasquilla and Lounibos 2015b).

In addition to opening the ventral tuft, Linley and Simmons (1981) suggest that active female transport is necessary for sperm storage. Contrary to Jones and Wheeler (1965b), they asserted that sperm locomotion alone cannot move sperm into the spermathecae as quickly as has been observed. Linley and Simmons (1981) argue that the rate of storage in the spermathecae is impossible given the measured sperm swimming speed (25 μm/s) versus the minimum diameter (1.2 μm) of the spermathecal ducts. According to their morphometric calculations, the only way for sperm to reach the spermathecae as quickly as they do is if females actively moved them. Yet Jones and Wheeler (1965b) noted no contractions of the spermathecal ducts (even though the ducts are surrounded by a helical musculature), and that the

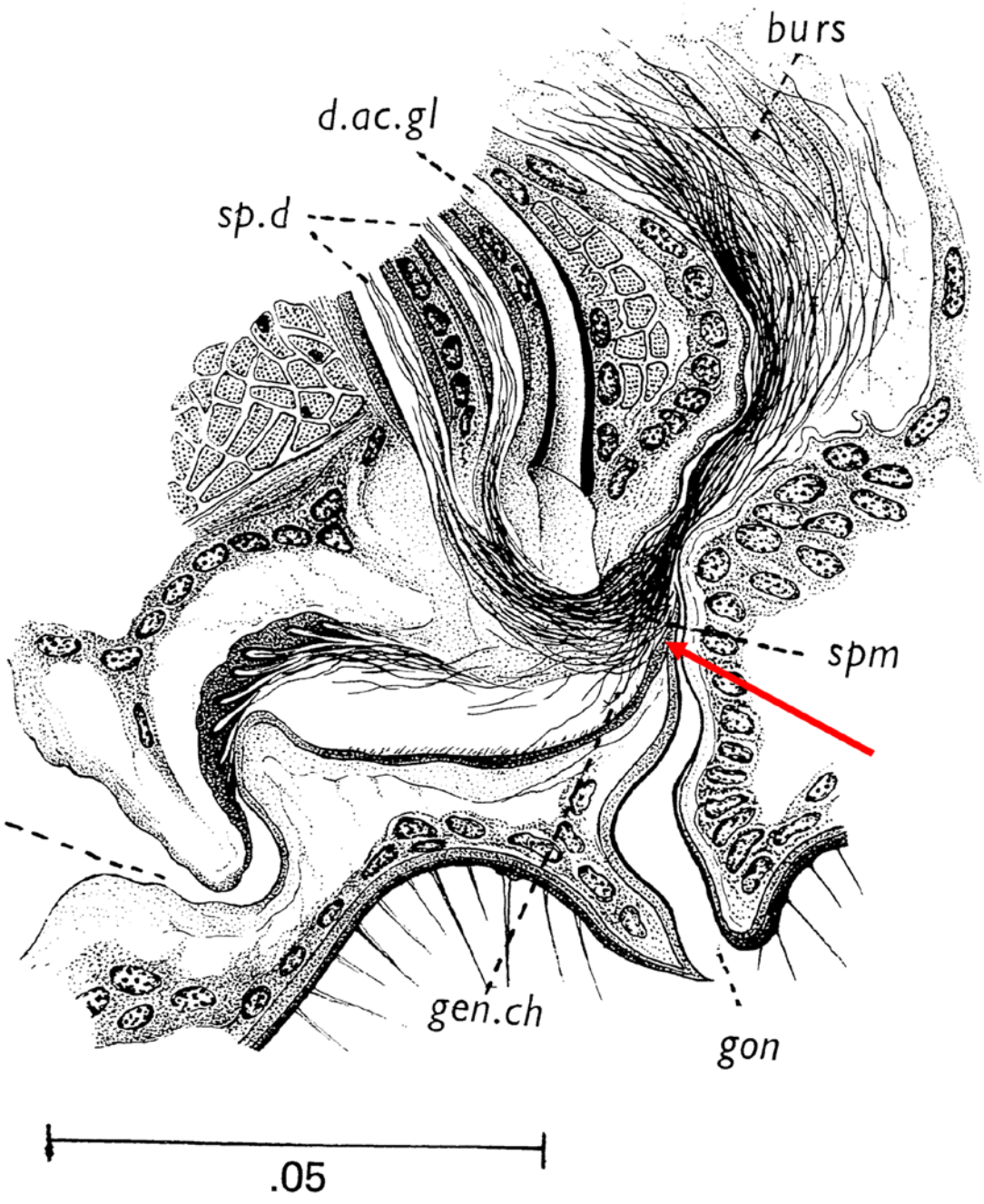

Figure 2.5. Sagittal section of the *Aedes aegypti* female reproductive tract (Jobling and Lewis, 1987). Red arrow indicates the ventral tuft, proposed by Spielman (1964) to act as a valve that guards access to the spermathecal ducts. Scale bar, 50 μm; burs, bursa; d. ac. gl, female accessory gland duct; gon, gonotreme; sp. d, spermathecal ducts; spm, sperm. Used with permission from the Wellcome Trust (London, UK).

spermathecal volume was static. Therefore, Linley and Simmons (1981) concluded that rapid fluid transport out of the spermathecae was the most likely mechanism,presumably accomplished by the spermathecal glandular cells. Linley (1981) concluded the same mechanism in the biting midge, *Culicoides melleus*, based on a similar argument. Clements (1999) endorses this idea, suggesting that the microvillar structure of the spermathecal glandular cells in *Ae. aegypti* is consistent with an ability to rapidly transport liquid. Further evidence for liquid export from the spermatheca is the fact that dyes injected into the bursa are sometimes observed in the spermathecae, suggesting that negative pressure draws in bursal contents (Jones and Wheeler 1965a). Exactly what triggers this process is unknown, but seminal fluid molecules may be involved since male-transferred seminal proteins and hormones play critical roles in modulating many other aspects of the female post-mating response (reviewed in Baldini et al. 2012).

Based on the above arguments, fluid transport likely plays some role in sperm transport, although we postulate that the role of sperm locomotion in the storage process may be underestimated. *Ae. aegypti* in vitro sperm swimming speed was measured in a saline medium (Linley and Simmons 1981), but, based on what is known in other insects (Curtis and Benner 1991; Yang and Lu 2011), mosquito sperm may exhibit different swimming behaviors in the reproductive tract, as described above. The rapidly spinning aggregates of sperm described by Jones and Wheeler (1965a), and shown in our video, are consistent with the idea of mosquito sperm being hyperactivated in the bursa before storage. Spermathecal ducts have a high elasticity due to their resilin content (Giglioli 1963; Clements and Potter 1967), which may

allow for expansion of the ducts as a mass of sperm passes through. Additionally, even though Jones and Wheeler did not observe contractions of the spermathecal duct, this possibility should not be discounted. Indeed, the musculature surrounding the spermathecal duct may pump sperm peristaltically, as suggested in *An. melas* (Giglioli 1963); the yellow dung fly, *Scatophaga stercoraria* (Hosken and Ward 2000); and the ant, *Crematogaster opuntiae* (Wheeler and Krutzsch 1994).

The seemingly coordinated, rapid whirling motion of sperm in the bursa is suggestive of sperm cooperation. Sperm likely encounter each other frequently, as females receive over one thousand sperm in a single insemination (Ponlawat and Harrington 2009), and these sperm are in intimate proximity to each other. The interactions of sperm and the potential for sperm cooperation have been demonstrated in *D. melanogaster* by Yang and Lu (2011), who described sperm moving through the seminal receptacle in parallel, intertwined bundles. Sperm cooperation has also been documented in the fishfly, *Parachauliodes japonicus.* In these insects, sperm are transferred in bundles, with their heads glued together by a trypsin-degradable protein (Hayashi 1997). Bundles with more sperm had a higher aggregate velocity than bundles with fewer sperm (Hayashi 1998). While mosquito sperm do not form conjugated groups like other taxa (Hayashi 1998; Higginson et al. 2012), it is possible that mosquito sperm reduce drag by swimming in parallel, allowing for their faster storage in the spermathecae. Females of some mosquitoes may occasionally mate more than once (Boyer et al. 2012; Richardson et al. 2015), especially soon after their first mating (Degner and Harrington 2016). Therefore, males whose sperm fill the spermathecae quickly may reduce their risk of sharing paternity with a second male.

Maintenance and storage in the spermathecae

Once sperm reach the spermathecae, they are stored and maintained for the female's life. Remarkably, one study documented fertilization in more than 100-day-old females (Styer et al. 2007). While some authors suggest that fertility is reduced after multiple egg-laying cycles (Young and Downe 1982), the consensus remains that mosquitoes maintain and utilize sperm from a single insemination without increased sterility throughout their lifetime (Styer et al. 2007; Oliva et al. 2013; Shaw et al. 2014). The mechanisms and molecules that sustain viable sperm in mosquitoes long-term remain poorly understood, but this is an important area for future study.

Ae. aegypti sperm are often seen swirling around the perimeter of spermathecae (personal observation). *An. gambiae* sperm, on the other hand, were rarely motile in the spermathecae of recently mated females, although two thirds of the spermathecae contained motile sperm if they were dissected 24 h or more after mating, suggesting that sperm may become activated after they have been stored for some time (Verhoek and Takken 1994). One caveat is that both of these observations were made in spermathecae immersed in saline. In the honeybee, *Apis mellifera,* reduced concentrations of sodium and potassium can activate sperm (Verma 1973), so it is possible that the osmotic environment in mosquito spermathecae changes after dissection. Indeed, Verhoek and Takken (1994) reported that *An. gambiae* sperm become motile in the spermathecae after immersion in saline. Nonetheless, moving sperm were recently observed in the spermathecae of an intact *Ae. aegypti* female (Carrasquilla and Lounibos 2015a). How soon sperm become motile and what stimulates their movement in the spermathecae in vivo remains untested.

Maintaining live, motile sperm is energetically costly. Energy storage substrates, such as glycogen, have not been detected inside mosquito sperm, nor have they been described for sperm of *D. melanogaster* or other insects (Anderson and Personne 1970), so the energy needed to maintain sperm in storage is likely provisioned by the female. Mosquito sperm contain two long mitochondrial derivatives (Clements and Potter 1967; Bao et al. 1992), but the function of these organelles in aerobic metabolism is dubious. In *Cx. quinquefasciatus*, activity of cytochrome C oxidase, a necessary enzyme for oxidative phosphorylation, was weak in mature sperm (Bao et al. 1992). Perotti (1973) found a similar lack of activity in *Drosophila*. In the rove beetle, *A. bilineata*, cytochrome C oxidase was active in spermatozoa, but potassium cyanide, an inhibitor of this enzyme, did not reduce sperm motility (Werner et al. 1999). Therefore, anaerobic metabolism is most likely the mechanism that powers sperm, but whether or not the mitochondrial derivatives in mosquito sperm function to provide energy for sperm deserves further investigation.

Secretory cells associated with each spermatheca nourish sperm. These cells connect to the lumen of the spermatheca via ductules, and form a gland situated around the juncture of the spermathecal duct and capsule. Their ultrastructure has been extensively characterized by both light and electron microscopy in *Ae. aegypti* and *An. aquasalis* (Clements and Potter 1967; Pascini et al. 2012; Pascini et al. 2013). The secretions of these glands have not been examined directly in mosquitoes, but proteomic studies of the spermathecal fluid in the honey bee, *A. mellifera*, may provide insight to mosquito spermathecal function. Honey bees, like mosquitoes, store sperm for life without later replenishment, and therefore the spermatheca of this

hymenopteran plays an analogous supporting role. Indeed, the proteome of spermathecal fluid in *A. mellifera* is enriched for energy metabolism enzymes (Baer et al. 2009). While male accessory gland secretions also produce metabolic enzymes, the network of enzymes produced by the female is more extensive. Further, the metabolic networks derived from males versus females show very little overlap (Baer et al. 2009). Therefore, energetic sustenance of sperm may be partially achieved by male seminal fluid in the short term, whereas female secretions likely take over in the long term (Baer et al. 2009). Glycoproteins and lipoproteins, which are present in the spermathecae of all Diptera examined to date, may act as energy sources for sperm (reviewed in Heifetz and Rivlin 2010).

In addition to requiring energetic nourishment, sperm must be protected from oxidative stress. One enzyme that provides this service in *An. gambiae* is heme peroxidase (Shaw et al. 2014). Expression of heme peroxidase in the spermatheca was up-regulated after mating, and females with RNA-interference-induced knockdown of heme peroxidase suffered partial infertility that worsened with successive batches of eggs. Controlling oxidative damage is important in other insects as well. Proteins with antioxidant function have been described in the spermathecal fluid of *A. mellifera* (Baer et al. 2009), as well as both the spermathecae and seminal receptacle of *D. melanogaster* (Prokupek et al. 2009; Prokupek et al. 2010), implying that minimizing oxidative stress is important for long-term sperm viability across insect taxa.

Sperm modification in storage

Do mosquito sperm undergo modifications while in storage in order for fertilization to take place? One clue to this question comes from a study that uncoupled the receipt of semen and sperm. Adlakha and Pillai (1975) surgically removed accessory glands or seminal vesicles from *Ae. aegypti* and *Cx. quinquefasciatus* to produce males that only transfer sperm or seminal fluid, respectively, during mating. Sperm from males without accessory glands were capable of reaching the spermathecae, but fertilization did not take place; fertilization was rescued in females that were subsequently mated to a male that only transferred seminal fluid. A comparable study in *D. melanogaster* reported similar results (Xue and Noll 2000). Although semen receipt is necessary for fertilization, the mechanism by which semen acts to facilitate fertilization remains unknown. It is possible that semen directly modifies sperm to prepare them for fertilization. Alternatively, it may trigger a physiological change in females that is necessary for fertilization to occur (e.g., enabling sperm release mechanisms).

Mammalian sperm become fertilization-competent while in the female through structural and molecular modifications. This process, called capacitation (Austin 1952; Visconti et al. 1995a), resembles semen-mediated activation of sperm motility described in mosquitoes. Both processes are calcium-dependent (Yanagimachi and Usui 1974; Thaler et al. 2013), and both result in protein tyrosine phosphorylation (Visconti et al. 1995a; Visconti et al. 1995b; Thaler et al. 2013). The capacitation process in mammals is initiated by removal of cholesterol from the plasma membrane (Visconti et al. 1999a; Visconti et al. 1999b). To our knowledge, cholesterol removal

has not been described in insect sperm, but some limited evidence suggests that the mosquito sperm glycocalyx undergoes structural modification while in storage (Ndiaye et al. 1997). Therefore, whether a process analogous to mammalian capacitation exists in insects remains uncertain but warrants investigation, and comparison to mammals may guide investigations of this phenomenon in mosquito sperm.

Release from the spermathecae

Sperm leave the spermathecae and travel down the spermathecal duct en route to their final destination, the egg. Sperm release is likely controlled by the muscles encircling the spermathecal duct, which act as a sphincter (Pascini et al. 2012). Indeed, the duct musculature is well-innervated (Clements 1999). The inside diameter and shape of the duct varies, but is a mere 1.2 μm and stellate in cross-section at its narrowest point (Clements and Potter 1967; Linley and Simmons 1981). Such a narrow passageway probably serves to limit the number of sperm released per fertilization. Curtin and Jones (1961) noted that spermathecae move "vigorously and repeatedly dorsally and posteriorward" while laying eggs, but it remains to be determined if this is due to contractions of the spermathecal ducts or a consequence of an egg moving down the oviduct.

Sperm release in other insects is controlled neurohormonally. In the locust, *Locusta migratoria*, octopamine induces spermathecal contractions involved in sperm release. An octopamine receptor, Octβ2R, is also present in the oviduct and spermatheca of *D. melanogaster*. Mutants without *octβ2R* lay few eggs, and any eggs

laid are not fertilized, suggesting that this receptor coordinates sperm release and ovulation (Li et al. 2015). Sex peptide, a male seminal fluid protein, also mediates sperm release in *D. melanogaster* (Avila et al. 2010). This peptide localizes to sperm tails (Peng et al. 2005), but must be cleaved from sperm in order for sperm to exit from storage; indeed, sperm of mutant males whose sex peptide binds irreversibly to sperm do not leave storage properly (Avila et al. 2010). No male-derived sperm-bound proteins have been identified in mosquitoes to date. Additionally, nothing is known about the specific neural signals that coordinate ovulation in mosquitoes; however, a recent neurotranscriptome of *Ae. aegypti* (Matthews et al. 2016) should assist future investigations of this critical reproductive process.

The number of sperm released for fertilization is an important factor in estimating reproductive potential of females, since releasing too many sperm per egg may deplete the sperm cache prematurely – a phenomenon that has been scarcely examined in mosquitoes. Harber and Mutchmor (1970) reported finding three sperm heads in the eggs of *Cu. inornata*, and Davis (1967) counted 6-10 sperm in the micropyle of *Cx. fatigans*. In *Cx. pipiens,* one or two sperm enter the egg, but rarely more (Jost 1971). This is in contrast to *D. melanogaster*, in which only one sperm penetrates the egg (reviewed in Hildreth and Lucchesi 1963; Loppin et al. 2015). It is possible that the number of sperm released depends on the number of sperm a female stores: A variable sperm allocation strategy has been demonstrated in the yellow dung fly, *S. stercoraria*, as females released more sperm when the number stored was high than when it was low (Sbilordo et al. 2009). Releasing more than one sperm per egg

may be adaptive if sperm are plentiful, since multiple sperm provides increased assurance of fertilization.

Entry into the egg

Fertilization in mosquitoes occurs as eggs are oviposited (Figure 2.3). Eggs protrude halfway from the gonotreme but pause briefly when the micropyle, a small opening for sperm entry on the anterior pole of the egg, aligns with the egress of the spermathecal ducts (Curtin and Jones 1961; Giglioli 1963). Similar alignment of sperm and micropyle was also recently visualized in *D. melanogaster* (Mattei et al. 2015) and is a strategy thought to give the female stringent control of the fertilization of each egg, preventing gamete wastage (Downes 1968; Bloch Qazi et al. 2003).

Sperm-egg recognition is probably mediated by interactions between sperm proteins and egg carbohydrates (reviewed in Mengerink and Vacquier 2001; Perotti et al. 2001). Two likely egg recognition proteins have been identified in *D. melanogaster* sperm. A mutant strain, c*asanova,* lacks β-N-acetylhexosaminidase on the sperm head and is unable to fertilize eggs, despite successful arrival of sperm at the egg (Perotti et al. 2001). The *Drosophila* micropyle contains α-L-fucose, and its sperm have the glycoconjugate enzyme α-L-fucosidase (Pasini et al. 2008; Intra et al. 2015). This pairing is conserved within *Drosophila*, whereas the same enzyme is absent from sperm of the closely related genus *Scaptodrosophila* (Pasini et al. 2008), indicating sperm-egg recognition mechanisms are taxon-specific. Another glycosidic enzyme, α-D-mannosidase, localizes to the *D. melanogaster* egg and has been suggested to further contribute to sperm-egg recognition (Perotti et al. 2001). α-D-mannosidase was also

identified in the semen proteome of *Ae. aegypti* (Sirot et al. 2011), indicating that some shared lock-and-key mechanisms could mediate mosquito gamete interactions – although these models require experimental support.

A laid mosquito egg contains an oocyte whose development has been arrested at metaphase I of meiosis (Jost 1971). Jost (1971) assumed that the presence of sperm activates the resumption of meiosis in *Cx. pipiens*, as is the case in most vertebrates (reviewed in Ducibella et al. 2006). Yet, a recent study demonstrated that unfertilized *An. stephensi* eggs are activated by immersion in distilled water. Yamamoto et al. (2013) used DAPI staining to show that meiosis resumed after the egg was immersed in water – even those eggs dissected from the ovaries. Whether this is the primary activating stimulus or acts as a fail-safe mechanism to resume development is unknown. Activation by immersion likely does not apply to all mosquitoes, especially since some genera, such as *Aedes*, generally do not lay their eggs in water (Clements 1999). Therefore, sperm's role in mosquito egg activation has not been fully illuminated, and activating stimuli may depend on the ecology of different taxa.

A conserved component of egg activation in animals is a calcium wave that propagates through the egg (Roux et al. 2006; Miao et al. 2012; Kaneuchi et al. 2015). In *D. melanogaster*, eggs are activated while they pass through the oviduct (Heifetz et al. 2001), when extracellular calcium is imported (Kaneuchi et al. 2015; York-Andersen et al. 2015). Given calcium's role in sperm motility, an intriguing possibility is that the sudden influx of calcium into the egg prior to sperm arrival assists in the sperm's entry via activation of motility, ensuring the entire sperm tail enters the egg (Karr and Pitnick 1996). Whether egg activation facilitates sperm's penetration of the

egg in mosquitoes has not yet been determined. Once safely inside, however, fertilization occurs and sperm's journey is complete.

Conclusions and perspectives

Many questions about mosquito sperm remain to be adequately answered (Figure 2.6), particularly since the phenomena studied to date are often described in only one species. Most detailed accounts of reproductive processes in mosquitoes are from *Ae. aegypti*, owing to its importance as a disease vector and its ease of culture in the lab. Similar investigations of sperm in other mosquitoes lag severely behind *Ae. aegypti*, even in the critically important *Anopheles* vectors of malaria. Given the differing reproductive tract morphologies between Culicinae and Anophelinae, descriptions of sperm biology may not always be generalizable across subfamilies. Therefore, future work should investigate murky areas of mosquito sperm biology in vectors other than *Ae. aegypti*.

Most studies of sperm movement have relied on in vitro assays that test sperm response to potential activating molecules or other conditions (Jones and Wheeler 1965b; Linley and Simmons 1981; Swan 1981; Thaler et al. 2013; Pitts et al. 2014). Such studies are important first steps for understanding what drives sperm locomotion, but the results of these studies are unlikely to be generalizable to in vivo conditions. Investigators should therefore consider the environment sperm are likely to face in vivo, paying particular attention to osmolarity, pH, viscosity, and spatial constraints. Work by Manier et al. (2010) and Yang and Lu (2011) provides excellent descriptions of sperm motility and locomotion within the *D. melanogaster* female reproductive

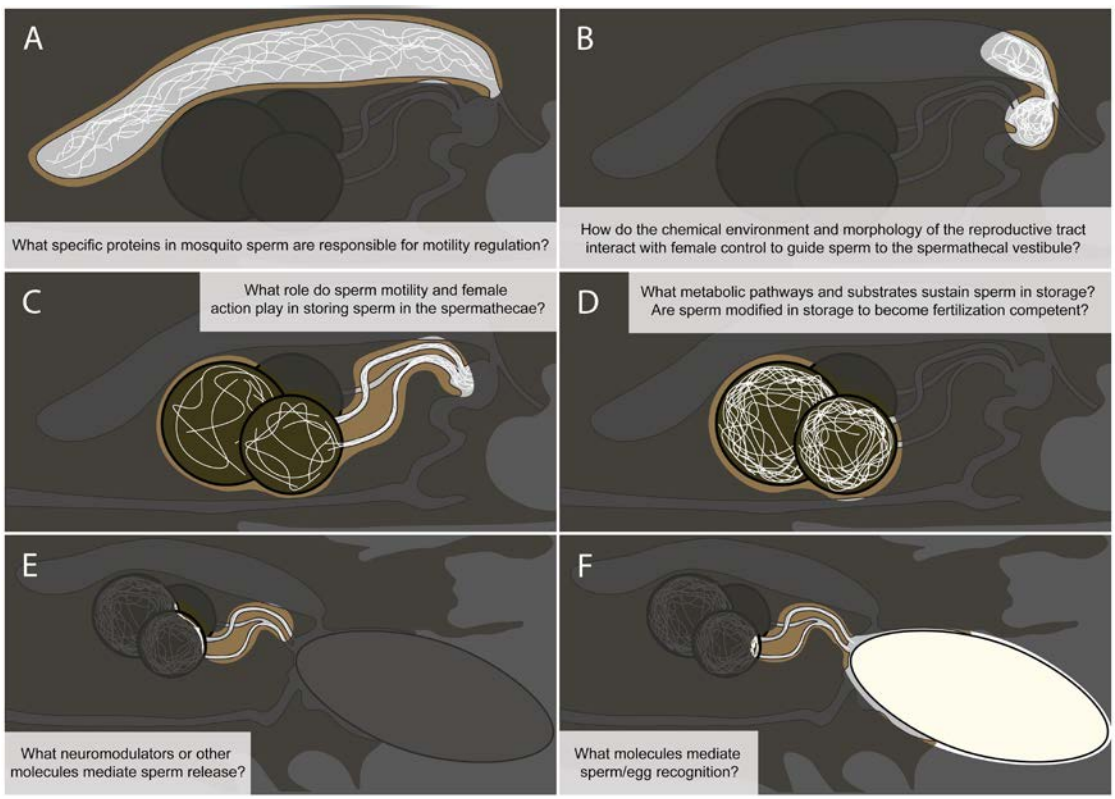

Figure 2.6. Analog of Figure 2.3, with text indicating unexplored, under-studied, and poorly understood aspects of mosquito sperm as they move through the female reproductive tract.

tract. Such elegant systems of visualizing sperm in vivo (Lu 2013) could be adapted to mosquitoes using lines with fluorescently labeled sperm, such as those produced by Smith et al. (2007). Other recent techniques in *D. melanogaster* may allow detailed studies of the molecular environment sperm experience. These include the methods of Kaneuchi et al. (2015) to visualize calcium fluxes in vivo, and of Heifetz et al. (2014) to map expression of specific molecules across the reproductive tract.

Research involving comparisons with other insects will shed light on under-studied aspects of mosquito sperm. The abundance of work on *D. melanogaster* sperm motility, storage, and usage is a substantial resource for investigations of mosquitoes.

As both mosquitoes and *D. melanogaster* are in the order Diptera, homology of proteins or processes may further our understanding of mechanisms utilized by mosquitoes. Spermathecal filling and sperm usage have also been well described in other flies, such as *S. stercoraria* (Sbilordo et al. 2009) and *Dryomyza anilis* (Otronen 1997; Otronen et al. 1997), and the morphological similarity of these species' spermathecae to those of mosquitoes may provide insights into the mechanisms of sperm storage and usage. Species that store sperm for life without replenishment, such as *A. mellifera* and other hymenopterans, may offer clues to how viable sperm can be maintained so efficiently for so long in mosquitoes. Finally, contrasting the unique 9 + 9 + 1 structure of the mosquito sperm axoneme to other insects may provide insight for the mechanics of flagellar motility.

In conclusion, the details of how mosquito sperm interact with the female on a mechanistic and molecular level is a largely unexplored, yet fascinating, field awaiting future investigation. Here, we have discussed the foundations for this evaluation and highlight areas that could yield the most insightful discoveries. Spielman (1964), Jones and Wheeler (1965a; 1965b), Clements and Potter (1967), and Giglioli (1963) provided a firm initial foundation of knowledge about mosquito sperm movement in the female, but they lacked adequate methods to delve deeper than basic morphological descriptions. Today, a vast array of genetic and molecular tools and resources has made it possible to answer questions that were once untouchable. For example, the sequenced genomes of *Ae. aegypti* (Nene et al. 2007), *Ae. albopictus* (Chen et al. 2015), *Cx. quinquefasciatus* (Arensburger et al. 2010), and 16 *Anopheles* species (Neafsey et al. 2015) provides a resource to mine for sperm-relevant genes that

are homologous to *D. melanogaster* or other organisms. Transcriptomics of reproductive tissues in *Ae. aegypti* (Akbari et al. 2013; Alfonso-Parra et al. 2016) and *An. gambiae* (Rogers et al. 2008; Baker et al. 2011) and proteomics of semen and sperm in *Ae. aegypti* (Sirot et al. 2011) and *Ae. albopictus* (Boes et al. 2014) provide databases from which specific genes of interest can be investigated. Finally, targeted mutagenesis has been accomplished in mosquitoes using rapidly advancing genome editing techniques, such as transcription activator-like effector nuclease (TALENs) (Aryan et al. 2013; Smidler et al. 2013), zinc-finger nucleases (DeGennaro et al. 2013; McMeniman et al. 2014), and, most recently, the CRISPR-Cas9 system (Dong et al. 2015; Kistler et al. 2015). Such resources and methods will allow the identification of proteins in sperm and in the female that play integral roles in activating sperm motility, guiding sperm through the reproductive tract, and enabling fertilization – ultimately placing a deeper understanding of mosquito sperm's journey from male ejaculate to the female's egg within our reach.

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CHAPTER 3

POLYANDRY DEPENDS ON POST-MATING TIME INTERVAL IN THE DENGUE VECTOR *AEDES AEGYPTI*[*]

Introduction

The mosquito *Aedes aegypti* transmits several pathogens to humans, the most important of which are the dengue (DENV) viruses and chikungunya virus. Globally, DENV causes significant disease, with an estimated 96 million clinically apparent cases annually,[1] 500,000 cases of which develop into dengue hemorrhagic fever, and 22,000 of which are fatal.[2] Chikungunya is an emerging viral threat and is responsible for epidemics worldwide.[3] While a commercial vaccine is emerging for DENV,[4] prevention of DENV and chikungunya virus transmission still relies heavily on vector control.

Several promising approaches for mosquito control involve field releases of modified mosquitoes that must mate with the wild population. Strategies examined to date involve reduction or replacement of the vector population with mosquitoes carrying disease-refractory traits. For example, *Ae. aegypti* carrying certain strains of the endosymbiotic bacteria *Wolbachia* are resistant to DENV infection.[5] In field trials, releases of such mosquitoes have been successful at replacing wild populations with *Wolbachia*-positive individuals.[6] Alternatively, control strategies may deploy males

[*] Presented with permission from The American Society of Tropical Medicine and Hygiene, with minor modifications from the originally published article: Degner, E. C., L. C. Harrington. (2016). Polyandry depends on postmating time interval in the dengue vector *Aedes aegypti*. Am. J. Trop. Med. Hyg. 94(4):780-785.

that carry lethal genetic cargo to functionally sterilize females. Such releases have been tested in several countries and show promise in reducing vector populations and disease burden.[7] Both approaches depend on successful mating interactions between released and wild mosquitoes, and thus the success of these strategies is intimately connected to mating behavior. However, some aspects of mating behavior, such as female mating frequency, remain poorly understood.

Normally, when *Ae. aegypti* females mate they become refractory to re-mating.[8, 9] This response can also be induced by injecting male seminal fluids into the hemocoel,[10, 11] but it is currently not known how soon the active molecule (or molecules) in seminal fluid takes effect. In *Aedes albopictus,* females can be re-inseminated if their second mating occurs within 40 min of their first.[12] In the malaria vector, *Anopheles gambiae,* males prevent polyandry by transferring the hormone 20-hydroxyecdysone in their ejaculate, but this molecule also has a delayed effect, with full refractoriness not induced until 1 – 2 d post-mating.[13] A similar effect of seminal fluid has been well-characterized in *Drosophila melanogaster*, in which refractoriness is induced by the seminal fluid protein sex peptide.[14, 15] Sex peptide does not immediately prevent a second mating,[16] and females are frequently re-inseminated within 4 h.[17] However, other mechanisms reduce insemination in this latent period, including the seminal fluid protein PEBII[17] and increased expression of a cuticular hydrocarbon that reduces attractiveness.[18]

In *Ae. aegypti,* polyandry has been documented in the laboratory,[8, 19, 20, 21] in a semi-field enclosure,[22] and with a small-scale study of a wild population.[23] How polyandrous behavior changes throughout a female's post-mating life is poorly

understood, but knowing when and why polyandry occurs has tremendous importance to population suppression strategies that deploy genetically modified males; if females re-mate most frequently after mating with modified males, they could undermine the success of inundative releases.[24] Some models of release strategy outcomes do not incorporate polyandry,[25, 26] but understanding baseline levels of polyandry is necessary for optimal development of a predictive modeling framework. Studying polyandry will also assist in predicting the spread of genes or traits in population replacement scenarios,[27] and it will provide insight into what drives male and female reproductive success.

Here, we examine two essential dynamics of female mating behavior: how quickly she becomes unreceptive to a second mating, and the extent to which she remains unwilling to mate over the course of her reproductive life.

Methods

Mosquitoes:

Two strains of *Ae. aegypti* mosquitoes were used: a Thai strain established from mosquitoes captured in Bangkok, Thailand, supplemented periodically with new field-collected individuals; and a DsRed transgenic strain with sperm that express the red fluorescent protein DsRed, emitting red light under green (557 nm) illumination.[28] All sperm from this strain were verified to display red fluorescence. Both strains were reared under similar conditions in a climate-controlled chamber at 28.0°C with 71.9 ± 9.5% RH and a daily light regime of 10 h light: 10 h dark with 2 h simulated dusk and dawn. After vacuum hatching, larvae were reared to pupae at a density of 200

larvae per 1 L of deionized water. Each tray of 200 larvae received 4 Cichlid Gold fish food pellets (Hikari, Himeji, Japan) to produce adults of uniform size (Supplemental Methods). Females eclosed in individual test tubes to ensure virginity; males eclosed in 8 L bucket cages, with any incidental females removed before 24 h.[29] Adults were held in separate cages by sex at a density of 200 adults per cage with access to 10% sucrose ad libitum. All mosquitoes used were 4-7 d old.

Virgin mating rate:

To establish a baseline mating rate using our 2 strains, 100 virgin Thai females were allowed to mate with DsRed males for 2 h at a density of 10 males and 10 females per 2 L carton. After 2 h, males were removed and mating rate was determined by checking females' reproductive tracts for fluorescence.

Onset of Refractoriness:

We tested the hypothesis that after a female's first mating, she gradually becomes refractory to a second mating over time. Females were allowed to re-mate at different time periods after their first mating to determine how the likelihood of re-insemination changes. Eight treatments corresponded to different post-mating time intervals during which females would not be allowed to re-mate: 0 – 2 h post-mating (hpm), 2 – 4 hpm, 4 – 6 hpm, 6 – 8 hpm, 8 – 10 hpm, 10 – 12 hpm, 14 – 16 hpm, and 20 – 22 hpm. Five replicates were conducted on different days: 3 in which all intervals were tested, and 2 additional days on which only the first 3 intervals were tested.

First, matings between Thai females and Thai males were observed. Virgin females were introduced singly or doubly into an 8 L cage with up to 15 males. Fresh virgin males were added to cages as the numbers became depleted. The total number of males in mating cages was not held constant over time because it did not impact the individual mating interactions that we carefully monitored and captured in this study. To encourage flight, the cage was knocked gently every time a female landed without coupling. Upon coupling, male and female pairs were observed closely for firm genital union. Those males and females that locked terminalia for longer than 8 s were considered successfully mated. The accuracy of this method was verified in a preliminary experiment by checking the spermathecae of females after their observed matings; 1 female out of 100 was not inseminated. After copulation terminated, both the male and the female were removed. All males and those females that mated for shorter than 8 s were discarded. Inseminated females were kept in groups of 10 in a 2 L carton, and the time at which each group was inseminated was recorded. To account for the effect of temperature and humidity on mating behavior, all matings were conducted in a climate-controlled environmental chamber under the conditions described above for rearing. After the appropriate post-mating interval, 10 DsRed males were added to each carton, and females were given the opportunity to re-mate for 2 h. To ensure that females in the 0 – 2 hpm treatment were allowed to re-mate immediately after mating, females in this treatment were directly placed into a carton of 10 DsRed males as soon as they were mated (less than 30 s after mating). This procedural difference was verified to have no effect on re-mating frequency (Supplemental Methods). All re-mating periods were conducted between 1100 and

1400 hours (with dawn at 0600 hours), and the exact time at which this period began was recorded. After the re-mating period males were discarded.

Females receive semen into their bursa, from which sperm travel to spermathecae (long term storage organs) within minutes.[30] If they receive a second insemination, they may or may not store sperm from the second male. Polyandrous females were defined as those that received a second insemination into their bursa, regardless of whether the second male's sperm were stored. To determine polyandry, females were anesthetized on ice and dissected in a drop of physiological saline. The reproductive tract was removed from each female, and the bursa was examined for DsRed fluorescence; any female that had fluorescence in her bursa was scored as re-mated. To verify that females were successfully mated by Thai males, the spermathecae of any DsRed-mated females were broken open by gently applying pressure from a coverslip, and sperm from each female were observed for fluorescence. Females with a red bursa and any wild type (non-red) sperm were considered polyandrous. Those with only DsRed sperm were considered to have never received a Thai male insemination, despite observation of copulation; these accounted for 1.7% of females (range 0.6 – 2.9% across treatments). This proportion is consistent with the failed insemination rate (1%) observed in females after observed matings.

Females that were not successfully inseminated by a Thai male were discarded from analysis. Re-mating cartons of 10 females were considered the experimental unit, and the proportion of re-inseminated females per carton was square root transformed to normalize residuals. A univariate general linear mixed model was constructed using the transformed data as the response variable. Explanatory variables included the post-

mating interval as a fixed factor, the time of day at which re-mating period occurred as a covariate, replicate as a random factor, and all factorial interactions between these terms. Although post-mating interval is a temporal variable, it was modeled as a categorical variable because the experimental design included 8 discrete treatments, and these intervals were not evenly spaced. The model was run iteratively; the term with the highest *P*-value was removed each time until all remaining terms were significant predictors ($P < 0.05$). Post-hoc pairwise comparisons were made using estimated marginal means with a Bonferroni correction and a significance threshold of $P = 0.05$. In most virgin cartons all females mated, and therefore these data did not fit a normal distribution. Therefore, comparisons of each post-mating treatment to virgins were made using one-sample *t*-tests, with the null hypothesis conservatively chosen as 0.8, the lowest proportion of mated females observed in a carton. Statistics were performed using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY2012).

Gonotrophic Cycles:

We tested the hypothesis that females become more receptive to a second mating after completing multiple gonotrophic cycles (i.e., taking multiple blood meals and producing several batches of eggs). Females were assayed for re-mating after 1, 3, and 5 gonotrophic cycles. To control for the possibility that the length of the post-mating time interval, rather than progression through gonotrophic cycles, may affect a female's mating behavior, parallel treatments were included that were fed only sucrose and did not lay any eggs. Previous authors have determined that nearly all 28 d old

Table 3.1. Sample sizes of gonotrophic cycles experiment. Number of females tested in each gonotrophic cycle treatment (GC) and its corresponding sucrose-fed control (S).

Treatment	Replicate A	Replicate B	Total
1GC	88	83	171
1S	96	85	181
3GC	83	98	181
3S	99	91	190
5GC	148	130	278
5S	137	93	230

virgin females are receptive to insemination,[20] so a control of the mating ability of old females was not included in this study.

Virgin Thai females were mated en masse to virgin Thai males by combining 200 females and 200 males in 8 L cages for 2 d. All mosquitoes were then cold-anesthetized and males were discarded. Females, now 4 – 5 d old, were separated into blood-fed and sucrose-fed treatments. Each gonotrophic cycle lasted 6 d and began with blood-fed females receiving a blood meal from ECD (Cornell IRB Human subjects activity exemption, FWA 00004513). After each feeding, females that took a blood meal were separated from those that did not. Unfed females were given another opportunity to feed several hours later, and those that still did not feed were discarded. Females were given oviposition substrate 4 d after blood-feeding. A solution of 10% sucrose was provided to both treatments ad libitum, except for 24 h prior to each blood meal, when it was replaced with deionized water in both treatments to facilitate feeding.

After 1, 3, and 5 gonotrophic cycles (6 d, 18 d, and 30 d, respectively), a subset of at least 90 females was removed from each treatment and placed into 2 L cartons

with 4 – 7 d old DsRed males at a density of 10 males and 10 females. After 24 h, males were removed and female reproductive tracts were examined for re-mating as described above. This experiment was replicated twice, for a minimum of 18 cartons total per treatment. Sample sizes are included in Table 3.1. No statistical tests were conducted on these data due to a lack of polyandry.

Results

Post-mating time interval significantly predicted re-mating frequency (Univariate general linear mixed model; $F = 9.031$; df = 7, 158; $P < 0.001$) (Table 3.2). The only other significant predictor was the day on which matings were conducted (Log-likelihood test; $\Delta G^2 = 8.154$; $P = 0.004$). We included this random factor in our final model to control for any potential effect of day. The estimate of residual covariance was 0.034, of which 0.006 (18%) was explained by the day effect.

After mating, 24 ± 3.0% (mean ± SE) of females were re-inseminated from 0 – 2 hpm. All subsequent intervals had a significantly lower rate of re-insemination than 0 – 2 hpm, ranging from 3.0 ± 1.4% (20 – 22 hpm) to 11 ± 2.6% (6 – 8 hpm). There was no significant difference between any intervals within 2 – 16 hpm. The interval with the least polyandry was 20 – 22 hpm (3.0 ± 1.4%); it was significantly lower than all intervals before 6 hpm, but not different from intervals after 6 hpm. Each post-mating interval had a significantly lower insemination rate than virgins, of which 96% were inseminated ($t < -12.31$; $P < 0.001$) (Figure 3.1).

No females that completed 1, 3, or 5 gonotrophic cycles were re-inseminated. Similarly, no sucrose-fed control females were re-inseminated, except for 2

questionable mosquitoes after 18 d post-mating. The bursae of these females had a faint fluorescence with small fluorescent particles scattered throughout the organ. The fluorescence was atypical of females inseminated by DsRed males, but was clearly above levels of background fluorescence. No fluorescence was observed in these females' spermathecae. Because only 2 females had this phenotype, they were unlikely to be biologically relevant and thus we excluded them from further discussion.

Table 3.2. Individual parameter estimates of univariate general linear mixed model. Parameters represent analysis of the square root transformed data of each treatment.

Parameter	**Estimate**	**95% CI**	***t***	***P***
Intercept	0.033	-0.091 ─ 0.156	0.556	0.586
0 - 2 hpm	0.428	0.310 ─ 0.546	7.161	0
2 - 4 hpm	0.218	0.105 ─ 0.332	3.807	0
4 - 6 hpm	0.19	0.076 ─ 0.305	3.293	0.001
6 - 8 hpm	0.176	0.048 ─ 0.303	2.72	0.007
8 - 10 hpm	0.097	-0.030 ─ 0.226	1.516	0.132
10 - 12 hpm	0.179	0.053 ─ 0.304	2.809	0.006
14 - 16 hpm	0.082	-0.050 ─ 0.215	1.227	0.222
20 - 22 hpm	0	N/A	N/A	N/A

Discussion

This study addresses 2 poorly understood but important aspects of *Ae. aegypti* mating behavior: the speed with which a female becomes refractory to a second mating, and the degree to which this refractoriness is maintained over multiple gonotrophic cycles. Our results support recent studies demonstrating that re-

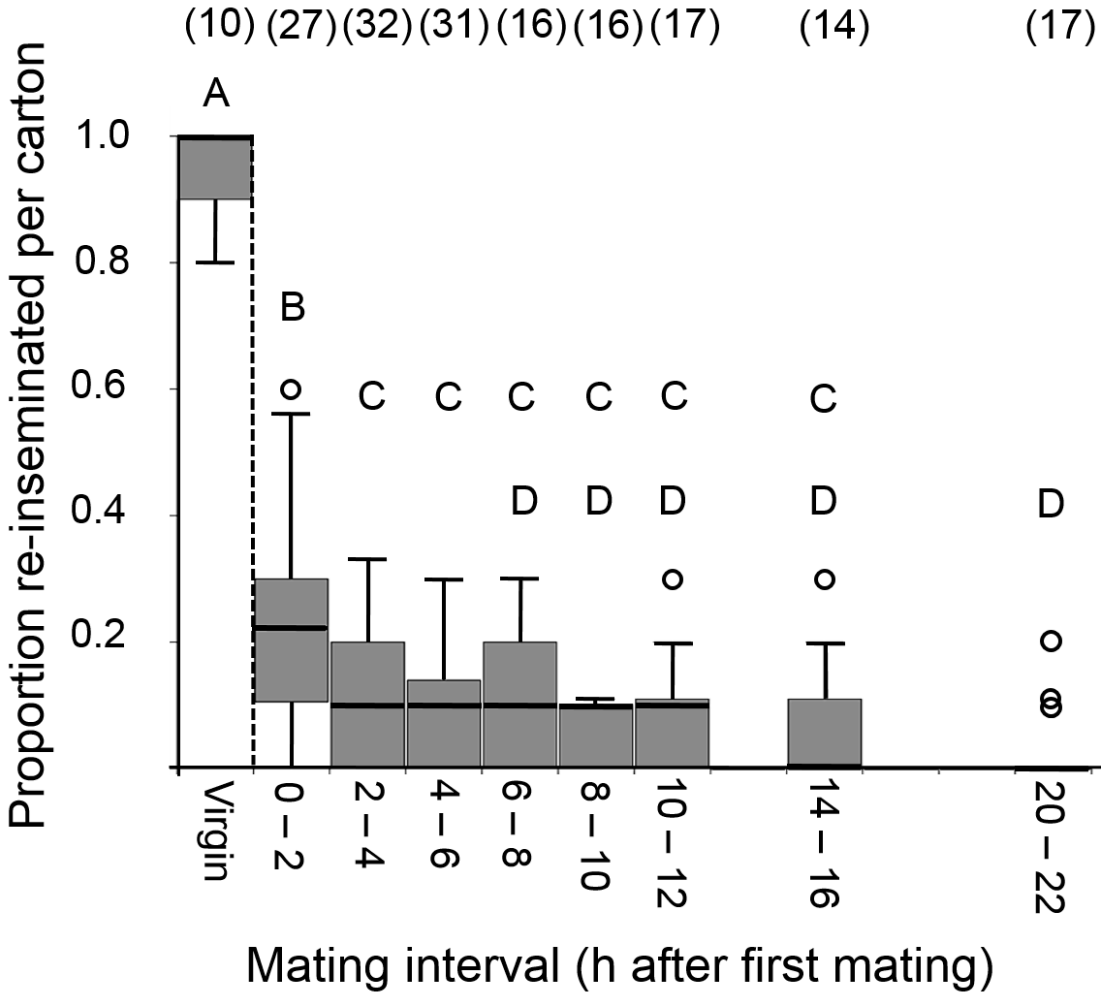

Figure 3.1. Onset of Refractoriness results. Proportion of virgin female *Ae. aegypti* mosquitoes inseminated (left of dashed line) and females re-inseminated at different post-mating intervals (right of dashed line). Virgins mated more frequently than females in all subsequent treatments (One-sample t test; $t < -12.31$; $P < 0.001$). Post-mating interval significantly predicts re-mating likelihood (Univariate general linear model; $F = 9.031$; $P < 0.001$). Only mated samples (right of the dashed line) were included in the model. Treatments with the same letter are not significantly different from each other (Post hoc pairwise comparisons with Bonferroni correction; $P > 0.05$). Untransformed data are shown, but analyses were performed on square root transformed data. Boxes and bold lines represent inner quartiles, whiskers are drawn using the Tukey method, and circles indicate outliers. Sample sizes (number of cartons containing 10 females) are included in parentheses.

insemination may occur in some situations.[22, 23] It is likely that reported cases of polyandry in these studies occurred before 22 hpm. In addition, we show that females do not mate after 6 d post-mating, regardless of progression through up to 5 gonotrophic cycles. Although the age of field-collected individuals is difficult to estimate, the majority of females likely do not survive beyond 5 gonotrophic cycles.[31] Therefore, we are confident that we encompassed the reproductive lifespan of female *Ae. aegypti* in our study.

Most females (76%) became refractory to a second mating within 2 h of their first mating. This behavioral shift may have occurred sooner, but we did not test earlier intervals. Females' shift to refractoriness undoubtedly benefits males by limiting the opportunity for sperm competition,[32] but the outcome for females is less certain. A polyandrous female may risk acquiring venereal pathogens,[33] or receiving an increased volume of seminal fluid may shorten her lifespan.[34] On the other hand, a female may benefit by receiving nutrition in the ejaculate[35, 36] or extra sperm if the first insemination was insufficient.[19] It is possible that females use an appraisal of their first male (based on his ejaculate, behavior, size, or other phenotype) to decide whether to mate again. Instances of skewing post-mating promiscuity based on first-male cues have been documented in other species, from solitary bees[37] to hamsters.[38, 39] If this is the case, a female may reap genetic benefits by increasing the proportion of her offspring that are sired by a second, more suitable mate.[40] While we did not test the proportion of a female's offspring sired by the first and second males, other authors have documented mixed-paternity offspring from females. [8, 23] Future investigations should quantify how multiple insemination affects first and second male

reproductive success. Whether a female ultimately re-mates is likely an integration of male manipulation and female interests and probably contributes to sexual conflict.

In our study, more females were refractory at 2 – 4 hpm (89%) than at 0 – 2 hpm, but the level of polyandry remained stable in intervals from 2 – 16 hpm (5.8 – 11%). We expected a steady decline in receptivity and were surprised to observe a static number of unreceptive females during the 2 – 16 hpm period. We hypothesize that refractoriness is induced in 2 steps; most females lose their willingness to re-mate shortly after their first mating, and the remaining receptive females become refractory gradually sometime after 16 h. Whether the mechanisms that elicit this 2-step response are the same or different remains unknown, but we caution against the assumption that all of a female's post-mating receptivity is dictated by a single mechanism. In *D. melanogaster,* before sex peptide induces long-term refractoriness, other molecules prevent females from re-mating.[14, 15] These include the seminal fluid protein PEBII[17] and a male-transferred pheromone that reduces female attractiveness.[18] Similarly, although long-term refractoriness is induced in *Ae. aegypti* by a seminal molecule (or molecules),[11, 41, 42] females may not be under strict control of this molecule before 16 hpm.

In the Onset of Refractoriness experiment, females mated most infrequently (3.0%) at 20 – 22 hpm, the last time interval we tested. We hypothesize that complete refractoriness sets in shortly thereafter. Helinski et al.[11] found that no females injected with male accessory gland extract were inseminated 2 d after injection, but they did not test earlier time points. Even if re-insemination were to occur at a similarly low rate after 22 hpm, polyandry at this time is unlikely to be biologically relevant.

Females typically mate in flight near the host[43] and are thus likely to feed soon after their first mating. Therefore, their mating opportunities would be limited while they rest, digesting their blood meal.

We found that once female refractoriness is established, it is complete, long-lasting, and independent of reproductive status; no females that completed up to 5 gonotrophic cycles were re-inseminated despite having the opportunity to re-mate, nor were sucrose-fed controls. This result agrees with Helinski et al.,[11] who found that no re-insemination occurred in sucrose-fed females up to 34 d after injection of male accessory gland extract. A similar result was found in *An. gambiae*, which also failed to re-mate after 5 gonotrophic cycles.[44] Female re-insemination in these disease vectors may be futile because irreversible post-mating changes in reproductive tract structure may prevent a female from storing a second male's sperm.[45] Furthermore, most *Ae. aegypti* females store a lifetime's worth of sperm from their first insemination,[46] and *Ae. albopictus* females do not suffer reduced fertility after 6 gonotrophic cycles.[12] Therefore, selective pressure for the ability to replenish sperm is probably weak.

Our results contrast with 2 reports that polyandry after multiple gonotrophic cycles is common in *Ae. aegypti*.[20, 21] The source of this discrepancy is uncertain but may lie in methodological differences. While we directly observed transferred semen from a second male, previous studies used the amount of seminal fluid in the bursa[20] or the transfer of radioactive isotopes from males to females[21] as indirect proxies for re-insemination. Another source of variation could be the strain of *Ae. aegypti* used. Both studies that found re-insemination used the Rockefeller strain of *Ae. aegypti*,

which has been maintained in laboratory colonies since the 1930s or earlier,[47] while we used mosquitoes derived from a recent field collection in Thailand. Laboratory evolution of polyandry has been documented in another insect,[48] and lab-adapted strain history could contribute to the differing results between previous studies and ours.

The experimental conditions in this study did not replicate the opportunities for re-mating that females experience in the field. However, carefully controlled studies like ours cannot be conducted in the field. In the wild, males typically intercept females as they approach a host to feed,[43] and thus female exposure to males is likely limited to when females are host-seeking. In our experiments, spatial constraints and duration of exposure to second males may have artificially inflated rates of polyandry. On the other hand, lack of host cues and actual opportunity to feed may have discouraged polyandry. Furthermore, the DsRed males used to assess polyandry were not as competitive for mates as their Thai counterparts (Supplemental Methods). Therefore, whether our experiments over- or underestimate re-mating is uncertain. Nonetheless, rates of polyandry in our study are similar to those found in a study conducted in a semi-field enclosure (14%)[22] and in a small-scale study of wild-caught females (6.25 – 14.6%)[23]. Knowing the frequency, timing, and ecological context of male-female encounters in a natural setting would aid our understanding of polyandry in the field.

While absolute frequencies of polyandry in our study may not be generalizable to all field populations, we nonetheless have described for the first time the dynamics with which refractory behavior is induced after mating and maintained over a female's

post-mating life. Our novel results will inform investigations aiming to identify molecules and pathways responsible for female post-mating behavior. In addition, understanding female mating frequency is an important consideration for vector control strategies that deploy genetically modified males or *Wolbachia*-infected females. Poor male quality or strain incompatibility might encourage females to mate again, and this could allow females to partially circumvent vector control efforts by mating with a second, wild type male. Increasing the number of released mosquitoes could compensate for the effect of non-random mating, and thus knowing how polyandry influences vector control outcomes is necessary for calculating how many mosquitoes to release. Therefore, this laboratory study provides a foundational understanding of when polyandry is likely to occur in *Ae. aegypti*, and our results will guide future assessments of re-mating frequency between strains relevant to specific release programs.

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CHAPTER 4

THE TIMING OF SPERM MODIFICATION, OVIPOSITION, AND FERTILITY IN THE MOSQUITO *AEDES AEGYPTI*[*]

Introduction

Mosquito reproduction is a promising target for controlling populations of mosquitoes that transmit human disease. While traditional mosquito control often aims to limit breeding sites[1] or kill larvae and adult mosquitoes with insecticides[2], interfering with reproduction after insemination could be a more efficient strategy for population reduction. Several such strategies are being developed or implemented. For example, in the sterile insect technique, irradiated males are released to mate with wild females (which are normally monogamous[3, 4, 5, 6]), but males transfer sperm incapable of fertilization (reviewed in [7]). A similar technique (being tested in the mosquitoes *Aedes aegypti* and *Ae. albopictus*) deploys males with *Wolbachia* bacterial endosymbionts, which, when mated with uninfected, wild females, prevent reproduction by inducing cytoplasmic incompatibility between sperm and egg (reviewed in [8, 9]). Genetically modified males that produce inviable offspring have been deployed to successfully reduce local mosquito populations[10, 11]. Finally, gene drive technologies are being developed to genetically sterilize females of the African malaria mosquito *Anopheles gambiae*[12]. Despite the promise of these vector control

[*] This chapter represents collaborative work with Jade M. Noble, Lena F. Kourkoutis, and Laura C. Harrington. JMN conceived of cryo-EM sample preparation techniques, conducted all imaging, and conducted some data analysis. ECD designed and implemented sperm motility, fertility, and oviposition assays, and analyzed their data. All authors conceived of experiments and critically edited the manuscript.

methods that manipulate post-copulatory reproductive processes, little is known about how sperm function in females after insemination.

A growing body of literature suggests that post-insemination modifications of sperm structure and motility are common phenomena across Animalia[13]. These modifications may serve to initiate or modify sperm motility, help sperm adapt to diverse environments encountered in the female reproductive tract, or prepare them for fertilization (reviewed in [13]). Sperm modifications are best described in mammals. For example, intracellular pH and calcium concentrations increase and ultimately lead to hyperactivated motility (reviewed in [14]), and in humans certain components of the plasma membrane are rearranged to facilitate exocytosis during fertilization[15]. In the osctracod crustacean *Mytilocypris mytiloides*, sperm are initially equipped with two protective layers but shed their outer coat once stored in the female[16]. The removal of this layer coincides with the onset of sperm motility and oviposition, suggesting that losing this coat may be required for female fertility. Similarly, eupyrene sperm (nucleated sperm that partake in fertilization) in the silkworm moth *Bombyx mori* also shed an outer sheath while in the female reproductive tract, whereas infertile apyrene sperm (without nuclei) do not. Because both sperm types are motile but sheath loss is specific to eupyrene sperm, it is inferred that loss of their outer sheath facilitates successful storage or fertilization[17]. Finally, micrographs of sperm of the mosquitoes *Culex quinquefasciatus*[18] and *Toxorhynchites brevipalpis*[19] suggest that removal of a glycocalyx, a cell coat containing a dense network of carbohydrate-rich molecules, occurs in these taxa as well, although it is unclear when such a removal occurs, what its purpose may be, or whether it is typical of all mosquito sperm.

Understanding when and where mosquito sperm are modified in the female reproductive tract may provide insight into how and why they are altered. Here, we describe the timing of sperm modification and processing in the mosquito *Ae. aegypti*—the primary vector of the viruses that cause dengue, yellow fever, and Zika. We used cryo-electron microscopy to investigate sperm ultrastructure before and at different times after insemination. This imaging technique allowed us to view mosquito sperm frozen in a near native state—without fixatives and stains that could alter their ultrastructure. We find that sperm are initially covered in a thick, highly organized glycocalyx, but within 12 h of storage within the female most sperm lack this coat. Using motility and fertility experiments, we attempt to correlate changes in sperm ultrastructure to function. Between periods of rapid motility in the bursa (where sperm are initially deposited in the female) and later in the spermathecae (where sperm are held for long term storage), we describe a period of inactivity shortly after insemination. To understand whether female reproductive capacity changes as sperm are modified, we also investigated how soon after mating females are able to fertilize eggs and lay them. We find that females both gain fertility and are stimulated to lay eggs while sperm are shedding their glycocalyx and after they have regained rapidly motility. Understanding the timing of physiological events is a crucial first step to identifying their underlying cellular and molecular mechanisms. Our results provide the foundation for future investigations of critical reproductive processes that could be manipulated for the purpose of vector control.

Results

Cryo-electron microscopy of mature Ae. aegypti *sperm reveals known and novel ultrastructural detail*

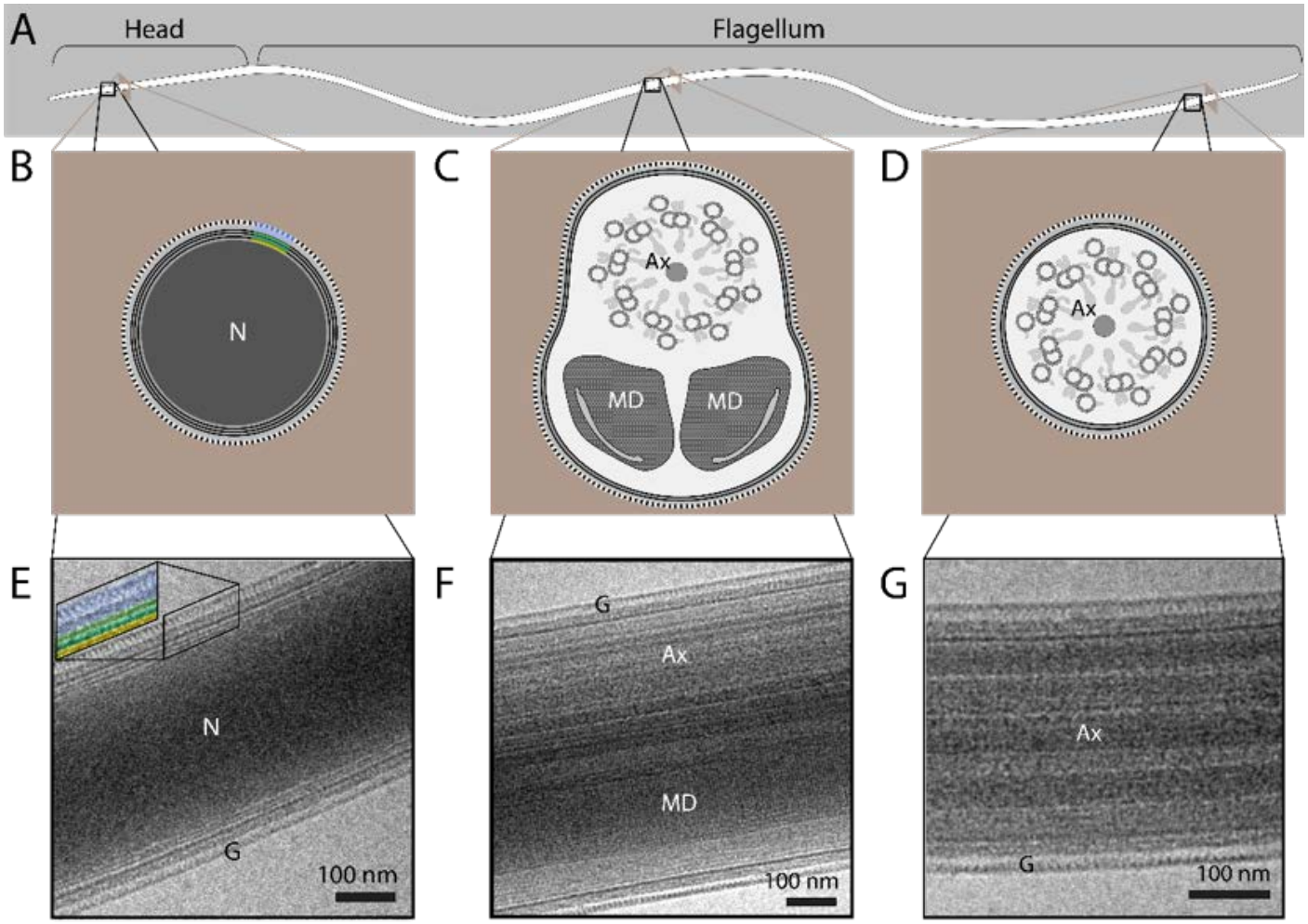
To understand baseline sperm morphology, we first dissected into saline sperm from the seminal vesicle of sexually mature male *Ae. aegypti* mosquitoes. Sperm in this organ have completely developed but have not yet mixed with seminal fluid contributions of the accessory glands. Because of these cells' extreme length-to-width ratio[20, 21], we show representative sections of different anatomical features, along with diagrams of their cross-sectional anatomy (Figure 4.1). In general, the ultrastructure of *Ae. aegypti* sperm at this stage is similar to that described in other mosquito genera[18, 19]. Mature spermatozoa were about 250 nm wide at each end, and 750 nm wide at their widest point, consistent with a previous study[20]. The needle-like 30 µm-long head (Figure 4.1A) is identifiable by its electron-dense nucleus with a homogeneous appearance, owing to its composition of tightly packed chromatin (Figures 4.1B, 4.1E). The flagellum is composed of two mitochondrial derivatives that run most of the length of the flagellum, and an axoneme that consists of microtubular rings and molecular machinery that power motility and extend nearly the full length of the flagellum (Figures 4.1C-D, 4.1F-G). The mitochondrial derivatives are identifiable by their paracrystalline protein structure[22] (Figures 4.1C, 4.1F, 4.2C), and the axoneme appears as striations running parallel to the length of the sperm (Figures 4.1C-D, 4.1F-G).

Mosquito spermatozoa are enclosed by a double plasma membrane ([18]; Figure 4.1B, 4.1E). This is best demonstrated in our micrographs in the sperm head, where

two plasma membranes and the nuclear membrane can be seen. Encapsulating the entire sperm cell is a 25 nm-thick glycocalyx (Figure 4.1B, 4.1E). This outermost layer appears to be anchored to the outer plasma membrane and is suggested in other mosquitoes to be deposited by secretory cells in the testes and *vas deferentia* after sperm have undergone individualization[19]. The glycocalyx appears to have three strata: the innermost and outermost being electron dense with an electron translucent layer separating the two (Figure 4.1E). Whereas micrographs of the glycocalyx in other mosquito sperm depict a homogeneous layer[18, 19], our stain-free cryo-EM approach revealed a striking, repeating structure to the glycocalyx in its outer two strata (Figures 4.2C-E), similar to that described in the grasshopper *Pezotettix giornae*[23]. In *Cx. quinquefasciatus*, the glycocalyx is proposed to be composed of carbohydrates based on labeling with various lectin-gold complexes[18], but the specific residues that comprise it remain undescribed. The double membrane + glycocalyx structure we observe in *Ae. aegypti* is consistent with micrographs of *Cx. quinquefasciatus* sperm, in which these layers can be seen in both transverse and sagittal cross sections[18].

We also note that sperm heads often had vesicles nestled among the condensed chromatin (Figures 4.2A-B). The majority of these appear to be located between the inner plasma membrane and the nuclear membrane, although at times it is difficult to determine their precise location. These vesicles were ovate, with major axes of 275 ± 80 nm and minor axes of 163 ± 40 nm (mean ± SD; n = 26 vesicles). Their contents are more electron-dense than the surrounding cytoplasm. In another mosquito, *Cx. tigripes*[24], similar structures have been suggested to originate from leftover membrane

Figure 4.1. (A) Diagram of full sperm length based on light microscopy (~250 µm long; width not to scale). (B, C, D) Diagrams of cross sections of sperm nucleus (B), anterior flagellum (C), and posterior flagellum (D) are based on previous electron micrographs of sperm from *Ae. aegypti* (Clements and Potter 1967), *Culex quinquefasciatus* (Bao and de Souza 1993), and *Toxorhynchites brevipalpis* (Ndiaye et al 1997). (E, F, G) Cryo-transmission electron micrographs in lateral view (this study) of sperm nucleus (E), anterior flagellum (F), and posterior flagellum (G). To identify surface structure, part of the surface in B and E (inset) are color coded to indicate the nuclear membrane (gold), inner and outer plasma membranes (green), and glycocalyx (blue). All images (E, F, G) are in lateral view. Ax, axoneme; G, glycocalyx; MD, mitochondrial derivative; N, nucleus.


A
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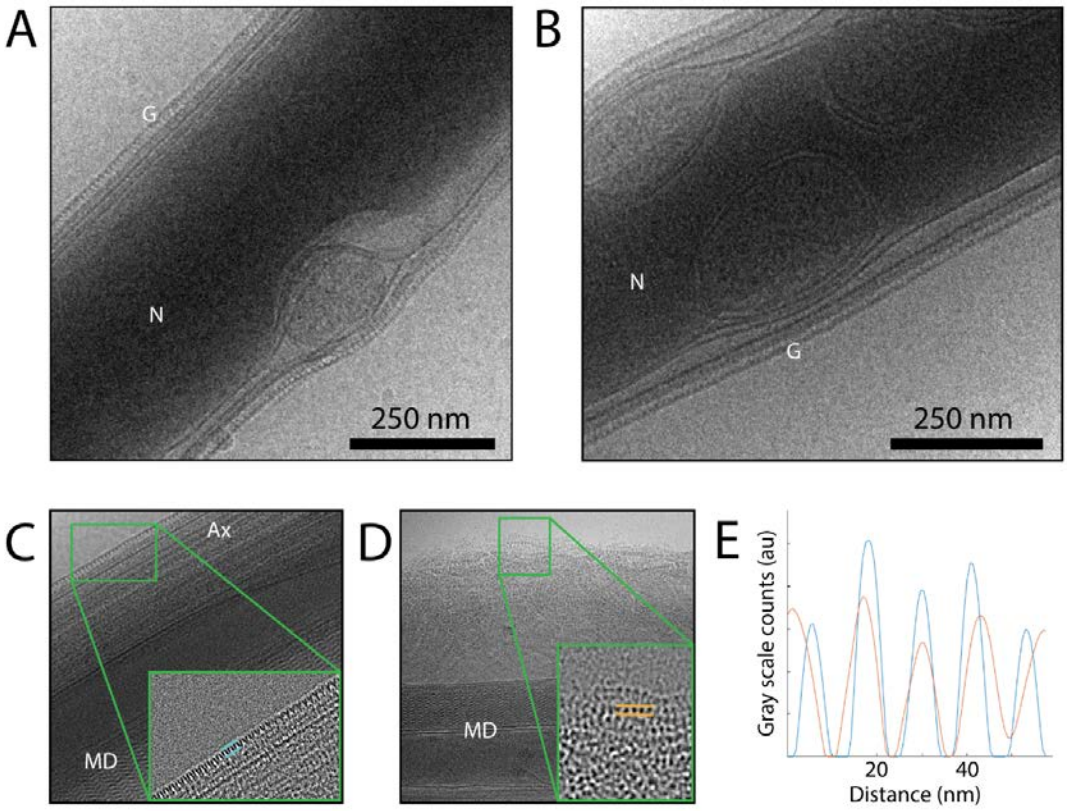


Figure 4.2. Novel observations of *Ae. aegypti* sperm morphology. (A-B) Vesicles nestled within the sperm head. (C-D) Glycocalyx with Gaussian blur ($\sigma = 2$; large images) and bandpass filter (< 3 px, > 10 px; insets) for intact glycocalyx (C) and glycocalyx peeling off (D). (E) Pixel intensity of the segment straddled by blue (C) and orange (D) parallel lines, highlighting the repeating structure that makes up the glycocalyx and assists in identifying the glycocalyx after it has dissociated from the cell (D). Ax, axoneme; G, glycocalyx; MD, mitochondrial derivative; N, nucleus.

as the nucleus is compressed during sperm individualization. Nonetheless, in light of the sperm/vesicle interactions described in *Drosophila melanogaster*[25] and mice[26], it is it is possible that vesicles in mosquito sperm have a more nuanced purpose than simply being a byproduct of spermiogenesis.

Sperm shed their glycocalyx within 24 h of storage in the female's spermathecae

Two previous studies of sperm from mosquitoes in other genera suggest that mosquito sperm lose their glycocalyx after mating[18, 19]. However, whether this modification occurs in *Ae. aegypti*, the timing with which it occurs, whether it occurs in all sperm or just a few, and its function remain uncertain.

Females are able to store sperm from one insemination for their entire life in spermathecae—a set of three rigid capsules that protect, maintain, and nourish sperm (reviewed in [12]). To compare sperm from the male's seminal vesicle to those in the female's spermathecae, we prepared sperm from the female at 24 h post-mating (hpm) by gently opening the medial spermatheca in saline using a minutien pin. Individual sperm were allowed to leave the spermathecae before flash-freezing them for imaging. Whereas all sperm from the male's seminal vesicle had an intact glycocalyx (Figure 4.3A-B), this layer had been stripped, along with the outer plasma membrane, from the whole length of all sperm harvested from the female's spermathecae at 24 hpm (Figure 4.3A-B). To determine whether the glycocalyx was removed as part of sperm's activation to motility during insemination, we next imaged sperm 3 min after insemination, when they are located in the bursa. In this organ, sperm are bathed in seminal fluid and bursal secretions and are activated to rapid motility[12]. At this point, sperm surface structure was indistinguishable from that of pre-insemination sperm from the male, with fully intact glycocalyces (Figure 4.3A-B). Finally, we examined sperm from the spermathecae at 4, 6, and 12 hpm. At these time points, an increasing number of sperm from the spermathecae did not have a glycocalyx (Figure 4.3C). By contrast, those with evidence of a glycocalyx displayed an intermediate state of

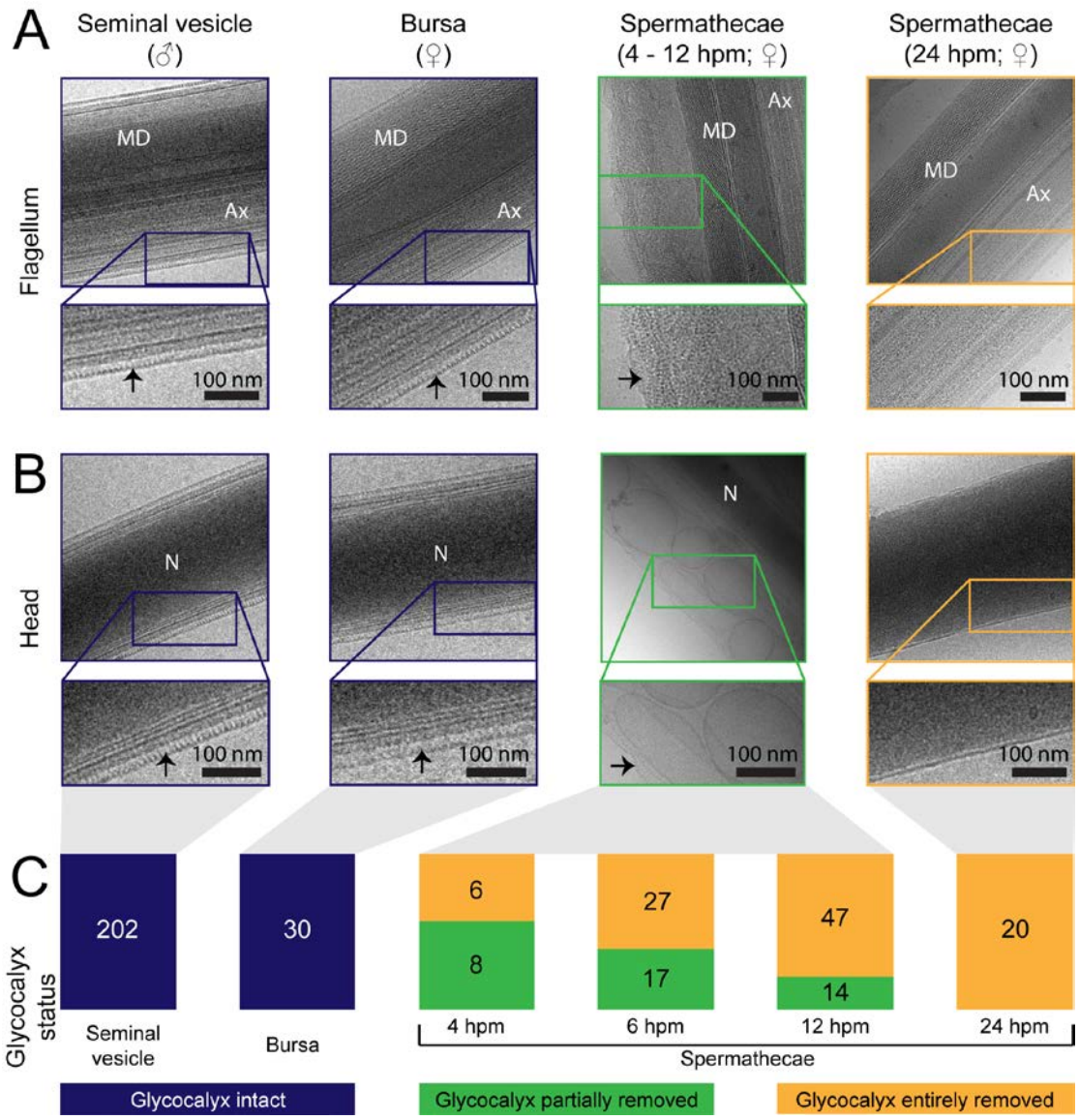


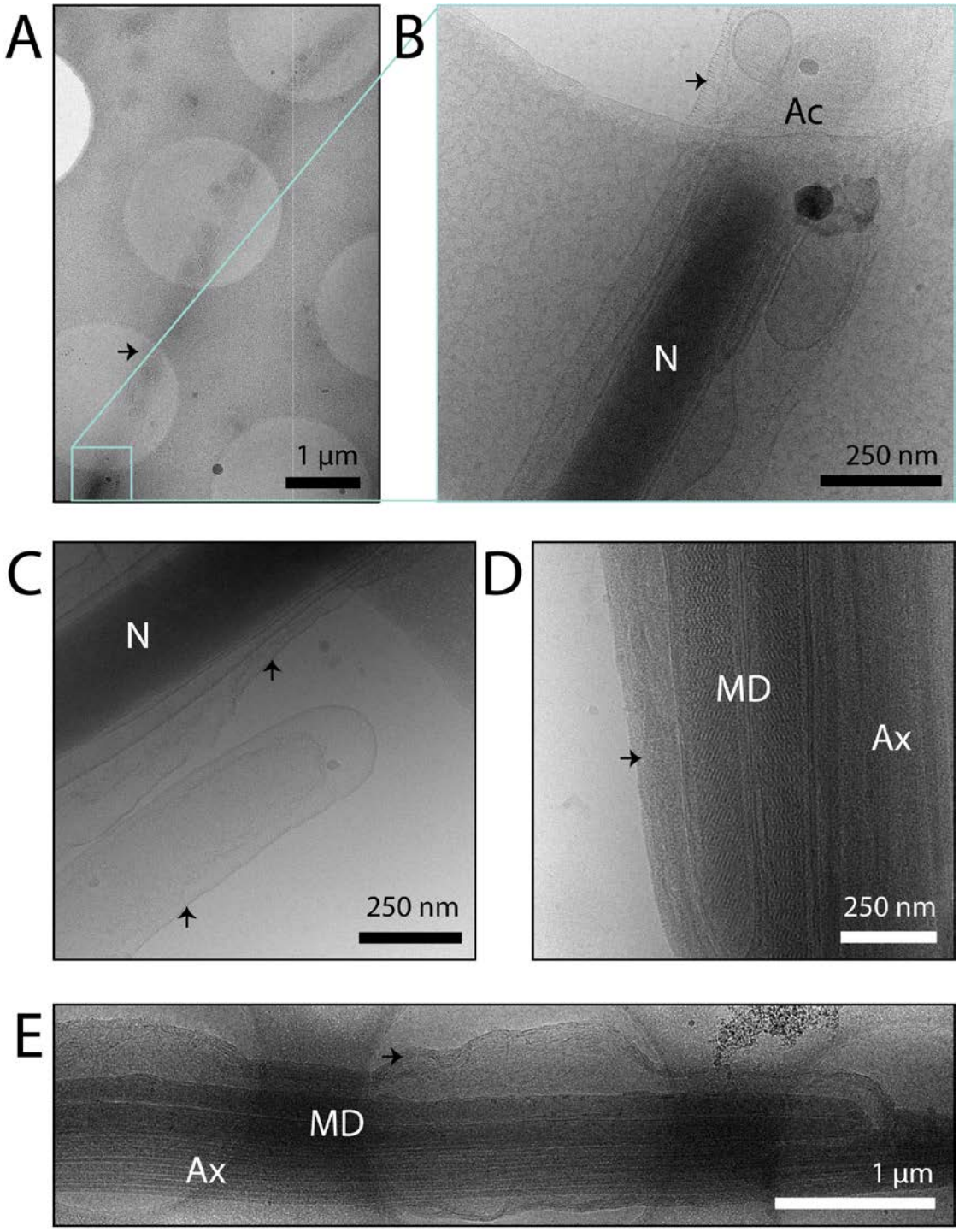
Figure 4.3. (A – B) Lateral view of sperm flagella (A) and heads (B) from seminal vesicle, bursa, spermathecae at 4 – 12 hpm, and spermathecae at 24 hpm. Insets show glycocalyx morphology and demonstrate intact glycocalyx in the seminal vesicle and bursa, glycocalyx dissociating from the sperm (4 – 12 hpm), and lack of glycocalyx at 24 hpm. Arrows indicate glycocalyx. (C) Proportion of sperm with glycocalyx intact (blue), with glycocalyx entirely removed (gold), or with intermediate state of removal (green) at different times before and after mating. Split bars represent proportion of total imaged sperm with given phenotype, and numbers in bars indicate number of sperm imaged with given phenotype. n = 11 males for sperm from the seminal vesicle, 3 females for bursa, 4 hpm, and 6 hpm, and 4 females for 12 hpm and 24 hpm. Ax, axoneme; MD, mitochondrial derivative; N, nucleus.

removal, with parts of the membrane appearing to have "peeled" off of the sperm (Figures 4.3A, 4.4D-E), or the glycocalyx sliding off of the head intact, with remnants of the outer plasma membrane inside the glycocalyx shell (Figures 4.3B, 4.4A-C). The repeating structure of the glycocalyx allowed for its identification during and after dissociation from sperm (Figure 4.2). We note that the number of sperm imaged from these time points was limited by the number that left the spermathecae (see below). If the presence of the glycocalyx in some way impedes sperm motility and detachment from the sperm mass during our sample preparation, then the proportion of sperm within the spermathecae whose glycocalyx is fully removed may be lower than the subset we were able to image.

Sperm are dormant for several hours at the same time the glycocalyx is being removed

Aedes aegypti spermatozoa display rapid motility while they are being stored[12] as well as within the spermathecae[27, 28]. However, while harvesting sperm from the spermathecae for ultrastructural imaging, we noticed that sperm (particularly those stored for time periods less than 4 hpm) were slow to exit the spermathecae shortly after mating. To test whether sperm exhibit altered motility after storage and during glycocalyx removal, we assayed sperm activity by gently cracking the chitinous, rigid covering of the spermathecae of females at different post-mating intervals and recording the emerging sperm (Video S1). Shortly after mating, sperm were sluggish and slow to emerge from the cracked spermathecae; they often only partially exited the spermathecae and did not escape to a free-swimming form. Those that dissociated from the sperm bundle showed compromised motility and weak swimming activity,

Figure 4.4. Images of the glycocalyx being sloughed from the sperm. (A and B) Empty glycocalyx shell (A), with inset (B) displaying the tip of the nucleus within the glycocalyx. (C) Tip of a glycocalyx shell alongside a nucleus whose glycocalyx is dissociating from the cell. (D and E) Glycocalyx peeling off the flagellum. Arrows indicate glycocalyx. Ac, acrosome; Ax = axoneme; MD = mitochondrial derivative; N = nucleus.


A
B
Ac
N
1 µm
250 nm
C
N
250 nm
D
MD
Ax
250 nm
E
MD
Ax
1 µm

with few traveling far from the spermathecae. However, with increasing time post-mating, sperm became more active, and upon cracking, escaped the spermathecal capsules faster (Figure 4.5A). As a proxy for overall activity within the sperm mass, we calculated the time required for 20 sperm heads to emerge from the ruptured medial spermatheca. Twenty sperm exit time initially decreased with increasing time post-mating (segmented linear regression; $df = 55$, $t = 5.89$, $p < 0.001$; Figure 4.5B; Table 4.1) and reached a minimum at 8.27 ± 1.9 hpm (breakpoint ± 95% CI), after which time no significant change in 20 sperm exit time was detected ($t = 1.78$, $df = 71$, $p = 0.08$; Figure 4.5B; Table 4.1). Variation in this metric, particularly at early time points, could be partially explained by the fact that females may receive between 400 and 4000 sperm when they are inseminated[29].

Oviposition and fertility plateau within 24 h of insemination

Given this timeline for sperm modification, we investigated whether other reproductive processes follow a similar timeline. To test how soon females lay eggs after mating, we blood fed virgin females, waited for them to produce eggs, and mated females at staggered intervals for 24 h. We then gave all females a 2 h oviposition period, in which they were given a moist substrate on which they could lay eggs. Finally, we attempted to hatch all eggs that females laid to assess whether females were fertile.

Post-mating interval strongly predicted oviposition. Oviposition prior to 12 hpm was sporadic and did not differ significantly from oviposition by virgin females. However, a stark increase in oviposition was observed after this time (Generalized

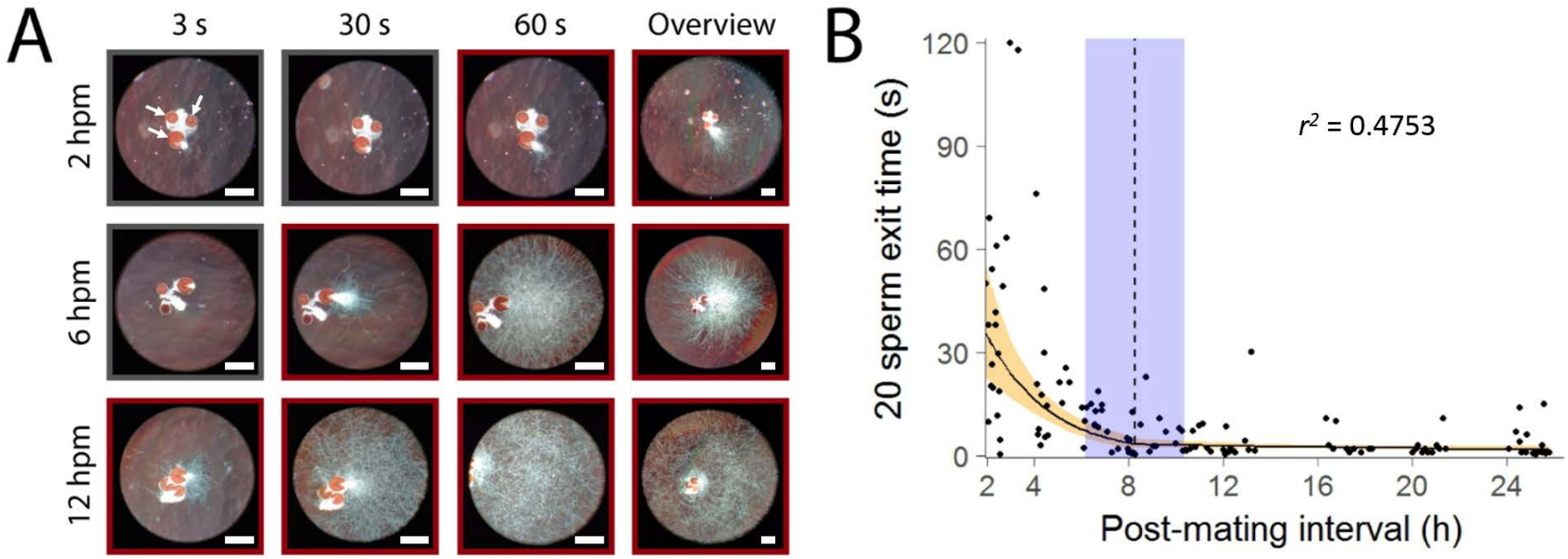


Figure 4.5. (A) (A) Spermathecae (indicated by arrows in top left image) from representative females cracked in saline at 2, 6, and 12 hpm, and imaged at 3, 30, and 60 s post-crack. Overview taken after 120 s post-crack. Boxes in red indicate samples in which 20 sperm have begun to exit spermathecae. Scale bars 200 µm. For examples of motility at different times post-mating, see Video S1. (B) 20-sperm exit time of females' spermathecae dissected between 2 and 26 hpm (n = 130). Solid line and accompanying orange ribbon indicate segmented linear regression with one breakpoint and 95% confidence interval; dashed line and blue ribbon indicate breakpoint of segmented linear regression and 95% confidence interval. Before the breakpoint (8.27 ± 1.91 h), 20 sperm exit time decreases significantly (t = 5.89, df = 55, p < 0.001), whereas after this point there is no significant decrease in 20 sperm exit time (t = 1.78, df = 71, p = 0.08), suggesting that sperm reach their maximum motility at the breakpoint. r^2 is the adjusted coefficient of determination for all data points. All calculations were performed on log-transformed data but are plotted in untransformed coordinates.

Table 4.1. Parameters of segmented linear regression. Values represent log-transformed data.

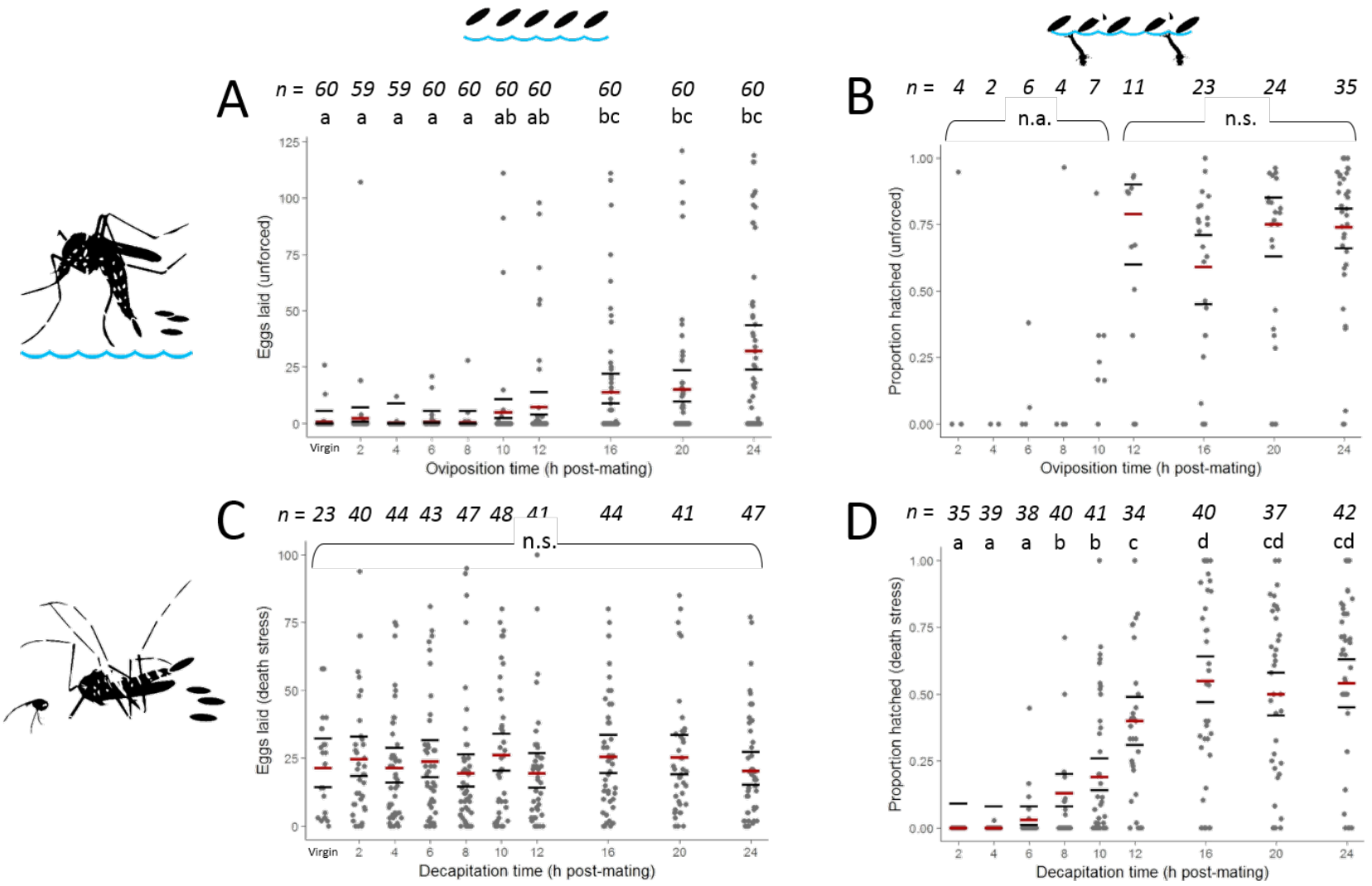
Segment	Slope ± SE	*t*	95% CI	*n*
First	-0.380 ± 0.064	5.89	-0.507 — -0.252	73
Second	-0.035 ± 0.020	1.78	-0.074 — 0.004	57

linear model (GLM); df = 9,588, F = 15.05, $p < 0.001$; Table 4.2; Figure 4.6A). From 12 – 24 hpm, post-mating interval did not significantly affect hatch rate (GLM; df = 3,88, F = 1.39, p = 0.25; Table 4.2; Figure 4.6B). Most females (88%) that laid eggs at or after 10 hpm produced viable larvae (n = 88 of 100), whereas prior to 10 hpm only 20% of egg-laying females (n = 4 of 20) produced eggs that hatched (Figure 4.6B). The receipt of seminal fluid, whether from normal mating or experimental intrathoracic injection in virgins, has been shown to stimulate oviposition[30, 31], demonstrating that oviposition is regulated hormonally and not necessarily by a female's ability to fertilize eggs. Given this disconnect between the capacity to fertilize and oviposition behavior, we reasoned that some females may be capable of fertilizing eggs before they are actually triggered to lay them. To identify the earliest time at which females become fertile, we developed an assay to force females to lay their eggs regardless of their mating status. If gravid *Ae. aegypti* are given a death stressor, they lay as many eggs as they are able in rapid succession[32, 33, 34]. Fertilization occurs as eggs are being laid, and death stressed females will also fertilize eggs if they are able[34]. Therefore, we prepared and mated gravid virgins at 2 h intervals in the same way as above. However, instead of providing oviposition substrate for these

Table 4.2. Parameters for GLMs investigating oviposition by unforced and decapitated females.

Oviposition Experiment	Model response variable	Variables in model	Likelihood ratio $\chi 2$	*df1,df2*	*p*	*F*
Unforced oviposition	Number of eggs laid	Intercept	4.94	1,588	0.027	4.94
		Post-mating interval	135.5	9,588	< 0.001	15.05
	Number of hatched eggs (≥ 12 hpm)	Intercept	52.76	1,91	< 0.001	52.76
		Replicate	4.23	1,91	0.043	4.23
Death stress oviposition	Number of eggs laid	Intercept	1256	1,408	< 0.001	1256
		Post-mating interval	5.58	9,408	0.78	0.62
	Number of healthy eggs	Intercept	1125	1,357	< 0.001	1125
		Post-mating interval	21.20	9,357	0.012	2.36
	Number of hatched eggs	Intercept	328.2	1,337	< 0.001	328.10
		Post-mating interval	395.2	8,337	< 0.001	49.40
Comparison of death stress and unforced oviposition	Number of hatched eggs (≥ 12 hpm)	Intercept	48.17	1,244	< 0.001	48.17
		Oviposition method	20.90	1,244	< 0.001	20.90

Figure 4.6. Eggs laid and hatch rate of females laying eggs either of their own volition (unforced) or via death stress oviposition. Each plot includes virgins and females at nine different post-mating intervals, and each dot represents one female. (A) Number of eggs laid by unforced females; (B) proportion of eggs that hatched from unforced females that laid eggs; (C) number of eggs laid by decapitated females, and (D) proportion of eggs that hatched from decapitated females that laid eggs. Times that have at least one letter in common indicate no significant differences between time points (generalized linear models with Bonferroni-corrected pairwise comparisons; see text for model structure). Black lines represent 95% confidence interval, and red lines represent estimated marginal means. *n*, sample sizes; n.s., no pairwise comparisons were significantly different; n.a., groups were not included in models or pairwise comparisons due to low sample size.


A
n = 60 59 59 60 60 60 60 60 60 60
a a a a a ab ab bc bc bc
Eggs laid (unforced)
Virgin 2 4 6 8 10 12 16 20 24
Oviposition time (h post-mating)
B
n = 4 2 6 4 7 11 23 24 35
n.a.
n.s.
Proportion hatched (unforced)
Oviposition time (h post-mating)
C
n = 23 40 44 43 47 48 41 44 41 47
n.s.
Eggs laid (death stress)
Decapitation time (h post-mating)
D
n = 35 39 38 40 41 34 40 37 42
a a a b b c d cd cd
Proportion hatched (death stress)
Decapitation time (h post-mating)

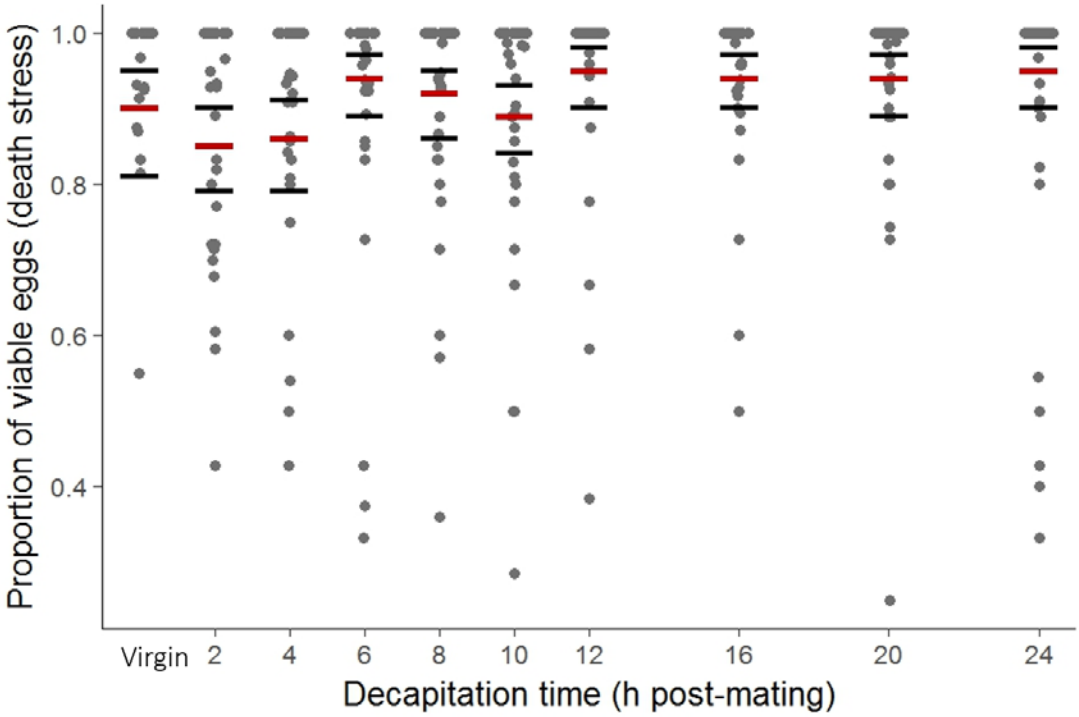


Figure 4.7. Viability of eggs laid via death stress oviposition by decapitated females at different times post mating. Each plot includes virgins and females at nine different post-mating intervals. Egg viability was defined as being convex in shape and fully melanized. Viability was significantly predicted by post-mating interval (GLM; df = 9,357, F = 2.36, p = 0.012). However, no difference between intervals remained after Bonferroni multiple comparisons correction. Black bars represent 95% confidence interval, and red bars represent estimated marginal means. Sample sizes same as total eggs laid (Fig 4.6C).

females, we decapitated them to force oviposition. We recorded the number of eggs they laid, the proportion of those eggs that were viable (as identified by a convex shape and full melanization), and the proportion that hatched. Females at all time points (including virgins) laid a similar number of eggs (GLM; df = 9,408, F = 0.62, p = 0.78; Table 4.2; Figure 4.6C). In addition, all time intervals had high proportions of viable eggs, ranging from 85–95%. While post-mating interval was significantly

associated with the proportion of viable eggs laid by a female (GLM; df = 9,357, F = 2.36, p = 0.012; Table 4.2), no significant

difference in egg viability existed among time points after multiple comparison correction (Bonferroni-corrected pairwise comparisons, $p > 0.05$; Figure 4.7). Despite similar egg numbers and viability across all time points, the proportion that hatched drastically increased from 6 hpm to 16 hpm (GLM; df = 8,337, F = 49.4, $p < 0.001$; Table 4.2), after which time hatch proportions did not change significantly (Bonferroni-corrected pairwise comparisons, $p > 0.05$; Figure 4.6D).

Finally, we compared hatch rates of eggs laid via death stress and normal oviposition to assess whether oviposition method influenced hatch success. Because no more than seven unforced females laid eggs in any time point prior to 12 hpm, we only included 12, 16, 20, and 24 hpm females in this comparison. Oviposition method significantly predicted hatch rate (GLM; df = 1,244, F = 20.90, $p < 0.001$; Table 4.2), with unforced oviposition resulting in significantly higher proportions of hatched eggs (Figure 4.6B, 4.6D).

Discussion

Herein, we describe the timing of four previously undescribed events in *Ae. aegypti* reproductive physiology: (a) the loss of a sperm glycocalyx after insemination, (b) a period of sperm inactivity that coincides with the removal of the glycocalyx, and (c) the onset of female fertility and (d) oviposition behavior, both of which occur at the same time or shortly after sperm are modified (Figure 4.8). We discuss these

Figure 4.8. Timelines demonstrating the onset of facets of the post-mating response tested in this study. Green bars represent the proportion of sperm with a glycocalyx, degree of sperm motility in the spermathecae, proportion of eggs that are fertilized, and the number of females laying eggs before mating and for 24 h post-mating. Highest points on each bar are scaled to the maximum proportion (glycocalyx presence, fertility, oviposition) or value (sperm motility) observed in this study. Curves are based on values measured in this study and are smoothed to simplify their interpretation.

findings in the context of challenges sperm may face in the female reproductive tract and reaching their ultimate objective to fertilize eggs.

Sperm in the male's seminal vesicle are mostly inactive. However, during insemination, they are deposited in the female's bursa (a temporary holding sac), where they almost immediately become rapidly motile[12]. The glycocalyx may function to protect sperm prior to storage (reviewed in [13]). While no threats to mosquito sperm have been empirically demonstrated during their ~5 min stint in the bursa, evidence from mosquitoes and examples from other systems suggest that sperm may experience

hazards shortly after insemination. For example, mosquito seminal fluid is loaded with proteases and peptidases[35, 36, 37, 38]. While some proteases likely function to activate sperm motility[39], it is possible that the catabolic cocktail in seminal fluid would be detrimental to an unprotected cell. In *Drosophila pseudoobscura*, a proportion of sperm are killed within 30 min of insemination, likely due to female reproductive tract secretions[40]. In mammals, sperm are equipped with a thick outer glycocalyx that is covered in glycoproteins and sialic acid residues that mask and defend the cell from the female immune system[41, 42]. The *Ae. aegypti* glycocalyx resembles that of orthopteran sperm[23], and a similar loss of the glycocalyx has been described in members of this order[43, 44]. Understanding the molecular composition of the glycocalyx in mosquitoes and the hazards sperm face after insemination may illuminate the function of the glycocalyx.

Once in the spermathecae, sperm activity drastically slows after a period of extreme motility in the bursa, in agreement with previous observations in *Ae. aegypti*[28] and *An. gambiae*[45]. While seminal fluid is assumed to nourish sperm while they are in the bursa, the spermathecae and their associated secretory cells likely take over this role after sperm are stored (reviewed in [46]). We propose that sperm's dormant period may occur due to a lag in the time it takes for spermathecal secretory cells to begin providing sustenance to sperm. It is also possible that sperm initially form a tangled mass in storage but disentangle themselves as they lose their glycocalyx and become motile. In support of this hypothesis, we note that sperm at 2 hpm in Video S1 appear moderately motile but disorganized and unable to escape the sperm mass, whereas at 6 hpm, sperm heads spill out as if they were oriented in the same plane and direction,

and sperm are able to escape the sperm mass with ease. A similar progression of events has been observed in the fungus gnat *Sciara coprophila*[47].

Sperm's move to the spermathecae may also involve being nourished by a new suite of metabolic pathways. In honeybees, seminal fluid and spermathecal fluid have vastly different metabolic networks[48], and the abundance of certain metabolic enzymes in honeybee sperm differs between freshly ejaculated and stored sperm[49]. Could the loss of the glycocalyx (and perhaps plasma membrane) play a role in this transition? It is possible that sperm's original protective coat enables a degree of intracellular homeostasis that is required for sperm to produce their own energy, and that once sperm are in the nutritive spermathecae their energetic needs are provided exogenously. Spermathecal secretory cells likely also provide antioxidant services, as they do in *An. gambiae*[50], and such a thick outer layer may impede the provisioning of peroxidases and other protective enzymes to sperm. A deeper understanding of mosquito sperm metabolism, sustenance by the spermathecae, and sperm motility may shed light on the function of the sperm glycocalyx and the reason for its removal.

Because little oviposition occurred prior to 10 hpm, we developed an assay using death stress oviposition to force females to lay their developed eggs. While eggs laid in response to death stress appeared morphologically identical to those laid by unforced females, it remains a possibility that certain events during ovulation or fertilization are less efficient when a female lays all of her eggs in quick succession. For example, the female accessory gland (a small, bulb-like secretory organ whose duct is adjacent to the site of fertilization[51]) is thought to produce secretions that are released during oviposition[52, 53], and death stress oviposition may force eggs to be laid

prior to the replenishment of such secretions. In support of this hypothesis, overall hatch rate was higher in eggs laid from unforced females. Despite this potential inefficiency, laying and fertilizing eggs when faced with certain death is a natural response[32, 33, 34] and likely serves to maximize the fitness of females that drown while ovipositing. Therefore, we propose that death stress oviposition is a valuable tool with which to study reproductive mechanisms, particularly shortly after insemination. Almost no females were fertile with this method through 4 hpm, but from 6–16 hpm, fertility increased drastically. Given that this change coincides with sperm losing their glycocalyx and becoming motile, a causal link between sperm modification and fertility is not inconsistent with our data. However, such a conclusion is premature given these findings alone. Rather, this study provides the groundwork for future investigations of physiological prerequisites for fertilization.

When oviposition was unforced, it drastically increases after 12 hpm. What stimulates oviposition at this time period? One possibility is that once females are capable of fertilizing eggs, an as yet undescribed feedback mechanism signals readiness to fertilize eggs and initiates oviposition. However, oviposition is likely more tightly linked to a seminal fluid component(s) rather than the actual ability to fertilize eggs. Intrathoracic injection of virgin females with a homogenate of male accessory glands (the primary producer of seminal fluid) induces them to lay eggs[30, 31], despite never receiving sperm. A similar response to the receipt of seminal fluid molecules has been described for insects in several other taxa (reviewed in [54]). In addition, oviposition in our experiments did not always result in viable offspring, even when a female had been naturally inseminated. Therefore, a transferred seminal fluid

molecule likely plays the major role in stimulating oviposition, and our experiments suggest that its timing is tightly coordinated with when females become fertile. It remains a possibility that fertility plays some role in inducing oviposition, although if it does, its impact is likely minor.

While long term female monogamy is well-established in *Ae. aegypti*[5, 6], the reason that females do not regain receptivity remains unclear. The timing of the events in this study may help to explain the *Ae. aegypti* mating system. We have previously reported that some *Ae. aegypti* females mate more than once, but this most often occurs prior to 2 hpm, and only rarely occurs after 16 hpm[6]. It is conceivable that once sperm are stored, shed their glycocalyx, and become motile, conditions in the spermathecae are adjusted to sustain and protect sperm rather than transform them, and females can no longer accept and process a second cohort of sperm. Females may also shift their reproductive agenda to focus on taking blood meals and laying eggs once they are fertile. Manipulative experiments using forced copulation[28, 55], artificial insemination[56, 57, 58], or genetic manipulations of female behavioral pathways[59] may shed light on the proximate and ultimate reasons for long-term female monogamy in *Ae. aegypti*.

Our descriptions of post-copulatory, pre-zygotic sperm modification, fertility, and oviposition offer valuable insight into how mosquitoes reproduce. Knowing how and when sperm undergo changes may help to answer key questions regarding mosquito reproductive physiology, including how sperm motility is directed; how they are stored; which metabolic pathways sustain them; how they interact with seminal fluid and the female reproductive tract; and whether certain modifications are

prerequisites for fertilization. Identifying the molecular and cellular underpinnings of such processes may lead to the development of methods to manipulate mosquito reproduction for the purpose of controlling mosquito-borne diseases. Finally, understanding how sperm are modified to cope with potential hazards, interact with the female, and fertilize eggs is important for understanding the biology of many internally fertilizing organisms, including livestock and humans. Our description of *Ae. aegypti* reproduction may provide new perspectives for investigators of reproductive physiology in diverse organisms.

Methods

Rearing

All mosquitoes were *Aedes aegypti* Thai strain and reared as described in [6].

Plunge-freezing vitrification:

Using low retention pipette tips, 3 μL of sperm suspended in phosphate buffered saline designed to be isotonic to mosquito hemolymph (as described in [38]; hereafter "saline") were pipetted onto holey carbon-coated 200 mesh copper grids (Quantifoil Micro Tools, Jena, Germany; hereafter "TEM grids"). R2/1 Quantifoil grids with hole sizes of ~2 μm were chosen to increase cell coverage. The TEM grids were blotted from the reverse side and immediately plunged into a liquid ethane/propane mixture at liquid nitrogen temperature using a custom-built vitrification device (MPI, Martinsried, Germany). The plunge-frozen TEM grids were stored in sealed cryo-boxes in liquid nitrogen until used.

All sperm imaged were from males or females aged 5–8 d post eclosion (dpe) and were dissected in saline. To collect mature, pre-insemination sperm, the seminal vesicle of males was isolated in saline, torn open at the anterior end, and gently stroked with a minutien pin to release sperm into solution. The dense sperm cloud was immediately aspirated and transferred to a TEM grid. Sperm from the bursae were collected by observing a male mating to a virgin female, dissecting the intact bursa from the female 3 min after copulation, and rupturing the bursa to allow sperm to swim into solution prior to pipetting them onto the TEM grid. Spermathecal sperm were collected by dissecting spermathecae from mated females at 4, 6, 12, and 24 h post-mating. Spermathecae were cracked using a minutien pin, given 10 – 15 s to allow sperm to swim out, and emerging sperm were carefully pipetted onto the TEM grid. To ensure that this 10 – 15 s delay was not the cause of altered sperm ultrastructure, we conducted a control experiment in which mature sperm from the male seminal vesicle were prepared with this delay (n = 2 males), and we observed no morphological changes from seminal vesicle sperm prepared without delay (data not shown).

Cryo-Transmission Electron Microscopy

Cryo-TEM was performed on a Titan Themis (Thermo Fisher Scientific, Waltham, MA) operated at 300 kV in energy-filtered mode equipped with a field-emission gun, and either a 2048x2048 Ceta 16M (Thermo Fisher Scientific, Waltham, MA), or a 3838x3710 pixel Gatan K2 Summit direct detector camera (Gatan, Pleasanton, CA) operating in counted, dose-fractionated modes. Images were collected

at defocii of -1 μm and -3 μm. Images were binned by 2, resulting in pixel sizes of 0.51-1.09 nm. TEM grids were initially scanned at low magnification to locate sperm, and any sperm that were located were examined at high resolution, provided they were located over a hole on the TEM grid and not embedded in crystalline ice. In post-mating samples, care was taken to image as much of each sperm's length as possible to understand how surface morphology changes over the entire cell.

Motility Assays

Females 3–14 dpe were allowed to mate with males for an hour at a 1:1 ratio. After this mating period, males were removed and females were maintained in an environmental chamber set at 71.9 ± 9.5% RH and 29 ± 1.0°C until assayed for motility. Females were dissected in saline. Each female's spermathecal triplet was removed and transferred to 60 μL of fresh saline. A glass coverslip was gently placed over the spermathecae, and the uncracked spermathecae were placed on a compound scope with 200x total magnification and dark-field illumination. The largest (medial) spermatheca was cracked with a coverslip by slowly wicking saline from the side of the coverslip with a Kimwipe. Immediately after cracking, the Kimwipe was removed to leave sufficient saline for sperm to move and exit the spermathecae. Videos of the spermathecae and the sperm they contained were recorded from the time of cracking for 2 min. Any spermathecae that cracked before the video was recorded or that had tissue (oviduct or spermathecal ducts) that covered the location of the crack was removed from analysis. As an estimation of overall sperm motility inside the

spermathecae, the time taken for 20 sperm heads to emerge from the medial spermatheca was recorded.

Unforced oviposition

Virgin females 2 – 4 dpe were offered a blood meal from ECD's arm after having been starved of sugar for 24 h. Females that did not feed were given a second opportunity to feed later that day. Females that still did not feed were discarded. After feeding, females were given 10% sucrose *ad libitum* and held in an environmental chamber as described above and allowed to develop eggs. At 6 d post-feeding, 35 females were combined with 35 males and allowed to mate for 2 h, after which males were removed. Over the next 24 h, eight more groups of females were similarly mated at 2 or 4 h intervals prior to oviposition. The mating status of 177 females was verified by checking their spermathecae for sperm, indicating that 92% of females successfully mated. At 7 d post-feeding, all females were given 2 h to oviposit at the same time, such that the nine groups of females had 2, 4, 6, 8, 10, 12, 16, 20, or 24 h between their mating period and oviposition period. A virgin control group was also given the same opportunity to oviposit. Oviposition took place in a 454 mL cup with a 30 mL oviposition cup lined with wet paper towel as oviposition substrate and filled with 10 mL deionized (DI) water. After 2 h, females were removed and the eggs they laid were counted. After 1 d, water was removed from each cup, paper towels were allowed to dry until they were slightly moist, and egg sheets were placed in a container with wet paper towel to maintain 100% relative humidity. After 3 d, eggs were submerged with DI water and a pinch of ground fish food (Hikari, Himeji, Japan), subjected to vacuum

pressure for 30 min, and allowed 5 d more to hatch. Finally, larvae were counted. Each post-mating interval included 30 females, and this entire experiment was conducted twice with different cohorts of mosquitoes.

Death Stress Oviposition

Gravid virgins were prepared as described above for unforced oviposition experiments. Females were mated for 30 min at the same intervals and sex ratios as unforced females. In this experiment, rather than providing oviposition cups to females, their heads were removed with forceps at the appropriate post-mating interval, stimulating death stress oviposition. Decapitated carcasses were placed individually on top of a wet filter paper disk in the well of a 96-well plate. Each well was covered with masking tape and the plate was placed in a plastic bag with a wet paper towel. Females were allowed to death stress oviposit for 1 h, after which they were removed from the wells and the masking tape was replaced. All females' spermathecae were then dissected to verify that they had mated, and females that had no sperm were pooled into a virgin control treatment. To avoid mold growth, excess water was removed from each well 1 d after oviposition. Eggs were allowed 3 d to embryonate, after which they were counted and scored as either viable (convex, fully melanized, and normally shaped) or inviable (deflated, not melanized, or irregularly shaped). At times, females laid many eggs on top of each other, preventing their exact enumeration without damaging the eggs. In such cases, we estimated the total number of eggs by counting those that were visible and estimating how many were not visible. Wells in which mold grew were recorded. Occasionally, some eggs exhibited a

phenotype in which the whole chorion was melanized, but horizontal stripes that lacked tubercles gave portions of the egg a glassy black appearance. We also recorded these wells. After counting and characterizing the eggs in each well, wells were flooded with 200 µL DI water and returned to the environmental chamber for 7 d. After 7 d, eggs were subjected to vacuum pressure for 30 min to stimulate hatching, after which the total number of hatched larvae in each well was counted. This entire experiment was conducted twice with different cohorts of mosquitoes. Each replicate initially included 30 or 32 females per treatment.

Statistical analysis

Segmented linear regression to analyze motility was conducted in R (v. 3.4.2, Vienna, Austria). Models to analyze oviposition analyses were created using SPSS (IBM SPSS Statistics for Windows, v. 24.0, Armonk, NY).

Motility: Changes in sperm motility were assessed using linear regression, with log-transformed twenty sperm exit times as the dependent variable and post-mating interval (calculated by the beginning of grouped mating periods to the time of dissection) as the independent variable. The time at which we stopped recording was used for the twenty sperm exit time of two females (out of 130) whose sperm did not leave the spermathecae. Because our initial simple linear regression indicated that twenty sperm exit time reached a minimum near 8 hpm, we conducted a segmented linear regression (package: "segmented"[60]) to quantitatively assess where the sperm motility plateaued. Whether the slope of each segment differed significantly from zero was tested using two-sided one sample *t* tests.

Oviposition: All oviposition data was analyzed using generalized linear models (GLM). Models assessing the number of eggs laid by each female had a Poisson distribution with a log link function, and all models assessing egg viability or hatch rate had a binomial distribution with a logit link. Because all data were overdispersed, the Pearson Chi-square was used as a scale parameter in each model. Models began with a fully factorial design, followed by iterative removal of the least significant term until all terms were significant ($p < 0.05$). Final model terms are included in Table 4.2. To compare response variables at different post-mating intervals, post-hoc pairwise testing was conducted using two-sided least significant difference comparisons with a Bonferroni correction (Figure 4).

Unforced oviposition: To assess when females are stimulated to lay eggs, post-mating interval and replicate as a factor, and eggs laid as a response variable. All females were included in the analysis ($n = 59 – 60$ per post-mating interval). To test whether post-mating interval affects fertility, each egg's hatch status was used as the response variable, and post-mating interval and replicate were included as factors. Because no more than seven females laid eggs prior to 12 hpm (Figure 4B), only females at 12, 16, 20, and 24 hpm were included.

Death stress oviposition: All wells in which any mold grew were removed from analysis ($n = 128$). For analysis of the total number of eggs laid, all remaining females were included ($n = 418$), with 40 – 48 females per post-mating time interval. A GLM was constructed in the same way as for unforced oviposition. For analysis of the proportion of eggs that were viable, all females that laid at least one egg were included ($n = 367$), with 37 – 44 females per post-mating time interval. Each egg's

viability was used as the response variable, and post-mating interval, whether wells contained striped eggs, and replicate were included as factors. For analysis of fertility, only mated females with at least one viable egg were included (n = 346), with 37 – 44 females per interval. Whether each viable egg hatched was included as a response variable; factors included post-mating interval, replicate, and whether wells had striped eggs. Several wells (9 out of 367) had slightly more larvae counted than the number of eggs that were initially counted, due to the need to occasionally estimate egg number. In these cases, the difference between larvae and egg counts was at most 8, and was on average 2. Because all of these instances were in the 16, 20, or 24 hpm time points, we kept these females in our analysis to avoid skewing data for only later time points. To ensure that these data fit our model's structure, we forced the number of larvae hatched in these wells to the number of eggs that were initially counted.

Unforced vs. death stress oviposition: Hatch rate of egg-laying females from 12, 16, 20, and 24 hpm for both oviposition methods was compared using each viable egg's hatch status as a response variable, and oviposition type and post-mating interval as factors. All eggs laid via unforced oviposition were assumed to be viable after several egg sheets were examined and found to have no concave or unmelanized eggs.

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CHAPTER 5

PROTEINS, TRANSCRIPTS, AND GENETIC ARCHITECTURE OF SEMINAL FLUID AND SPERM IN THE MOSQUITO *AEDES AEGYPTI*[*]

Introduction

The mosquito, *Aedes aegypti*, is the most important vector of arboviruses globally, transmitting viruses that cause dengue (1), Zika (2), chikungunya (3), and yellow fever (4). Consequently, *Ae. aegypti* places a severe strain on public health infrastructure around the world (5). Despite decades of effort to control mosquito populations, *Ae. aegypti* continues to contribute to human disease epidemics. New and improved control strategies are needed to prevent future outbreaks and mitigate disease burden.

Some promising control strategies under development target reproduction to suppress mosquito populations. For example, sterilized males can be released to suppress populations by impairing reproduction by their wild mates (6, 7). Manipulating reproductive phenotypes may also provide a means of driving disease-refractory traits into a population (reviewed in 8). One such strategy employs the intracellular bacterium *Wolbachia*, which, when introduced into *Ae. aegypti*, induces cytoplasmic incompatibility that allows the bacterium to spread in a population,

[*] Presented with permission from the American Society of Biochemistry and Molecular Biology, Inc., with minor modifications from the originally published article: Degner, E. C., Y. H. Ahmed-Braimah, K. Borziak, M. F. Wolfner, L. C. Harrington, and S. Dorus. (2018). Proteins, transcripts, and genetic architecture of seminal fluid and sperm in the mosquito *Aedes aegypti*. Mol. Cell Proteomics 18:S6-S22. ECD prepared all samples and conducted some data analysis. YHAB conducted all transcriptomic, GO, KEGG, and chromosomal analysis. KB conducted all proteomic and orthology analysis. All authors conceived of and critically edited the manuscript.

potentially to fixation (9). Cytoplasmic incompatibility causes sperm of males with *Wolbachia* to be incompatible with uninfected females' eggs, whereas *Wolbachia*-positive females can reproduce with any male, regardless of infection status (reviewed in 10), giving *Wolbachia*-positive individuals a fitness advantage over their uninfected counterparts. This bacterium also blocks or reduces transmission of several viruses, including dengue (11) and Zika (12). Consequently, introduction of novel *Wolbachia* infections into vector populations is being explored as a transmission reducing strategy.

Designing mosquito control strategies that target reproduction requires an intimate knowledge of the underlying cellular and molecular mechanisms. Yet, only a few functions of proteins involved in mosquito reproduction have been described to date. For example, in *Ae. aegypti* seminal fluid proteins (SFPs) induce several physiological and behavioral changes in females, including refractoriness to future mating (13-18), stimulation of oogenesis (19), enhanced survival (18), and the ability to fertilize eggs (20). However, the molecular identity of active SFP components for this species remains elusive. Seminal fluid initiates sperm motility via the action of proteases in many insects (silkworm (21); water strider (22); *Culex* mosquito (23)), but the precise sperm proteins on which seminal fluid acts in *Ae. aegypti* have not been identified. Similarly, sperm-associated odorant receptors may control motility in *Ae. aegypti*, although the exact function and ligands of these receptors are unknown (24). Finally, the mechanism by which *Wolbachia* induces cytoplasmic incompatibility has not been described in *Ae. aegypti*, but *Wolbachia* proteins contained in sperm are hypothesized to be involved (25, 26).

Identification of sperm proteins and SFPs that are transferred to females during copulation is an important objective to enable future investigations into specific reproductive processes. Components of the transferred ejaculate include sperm and seminal fluid—both of which play vital roles in mosquito reproduction. An *Ae. aegypti* seminal fluid proteome was first reported by Sirot *et al.* (27) based on mass spectrometry analyses. That study described 93 putative SFPs transferred during mating. While not a primary focus of their work, they also identified 101 putative sperm proteins. Later work identified more than twice as many SFPs from *Ae. albopictus* using similar methodology (28). Proteome complexity of other insects' seminal fluid (reviewed in 29, 30, 31) and sperm (32-34) suggests that more proteins remain to be identified in the *Ae. aegypti* ejaculate. Here, we used tandem mass spectrometry (MS/MS) with greater sensitivity and the recently revised and expanded genome to build on the foundational work of Sirot *et al.* by identifying constituent proteins of both *Ae. aegypti* sperm and seminal fluid. Importantly, significantly increased coverage of the sperm proteome allowed more accurate differentiation between seminal fluid and sperm proteins in the mixed ejaculate sample. We also profiled the transcriptomes of the male accessory glands (MAG; before and after mating) and testes, the major source tissues for SFPs and sperm proteins, respectively. Our proteomic characterization represents a nearly four-fold expansion of putative SFPs and a more than eight-fold expansion in the *Ae. aegypti* sperm proteome. Our results yield insights into the molecular function, genome organization, regulation, and evolution of sperm proteins and SFPs in this important disease vector. Ultimately, these proteomes provide a basis for future studies of *Ae.*

aegypti reproduction and, potentially, a catalog of molecular targets for the development of novel mosquito control methods.

Experimental procedures

Rearing

Mosquitoes were derived from a laboratory colony of *Ae. aegypti* that was established from individuals collected in Bangkok, Thailand, in 2011 and supplemented with wild caught mosquitoes every 2 - 3 y. Mosquitoes were reared as described previously by Degner and Harrington (35). Briefly, eggs were hatched under vacuum pressure, and a day later 200 first instar larvae were transferred to rearing trays of 1 L deionized water with four Cichlid Gold fish food pellets (Hikari, Himeji, Japan) as diet (except in the case of ^{15}N-labeled females; see below). Pupae were separated by sex based on size, allowed to eclose individually in separate test tubes, and adults were transferred into single-sex cages with 10% sucrose provided *ad libitum*.

Sperm Isolation

Males used for sperm protein sample preparation were aged between 5 and 8 days post-eclosion (dpe). Sperm were isolated from the seminal vesicles of each male to ensure a relatively homogenous population of mature sperm which have completed spermiogenesis. Seminal vesicle sperm likely also include proteins that could have been deposited on the sperm coat by the vas deferentia or seminal vesicle after sperm leave the testes (Ndiaye et al. 1997). Males were dissected in physiological saline

(133 mM NaCl, 2.63 mM KCl, 9.75 mM Na_2HPO_4, 3 mM KH_2PO_4, 2 mM $CaCl_2$, adjusted to pH 6.9; hereafter "saline"). The seminal vesicle was isolated from other tissue and consecutively transferred to two clean droplets of saline to remove any adherent fat body or other debris. Clean dissecting tools were used to transfer the seminal vesicle to a final droplet of saline where it was ruptured to release sperm. Sperm suspended in saline were transferred to a microcentrifuge tube on ice, and pooled sperm samples were flash frozen in liquid nitrogen every 2 h.

Two biological replicates included sperm combined from 400 and 470 randomly selected males, respectively. Pooled samples were centrifuged at 25,000 x g for 10 min at 4°C. The supernatant was removed, leaving 18 µL saline with the pellet. An equal volume of 2x Laemmli buffer + 5% β-mercaptoethanol was added to the pellet, and samples were solubilized by sonicating for 30 s, boiling for 15 min, and re-sonicating for 30 s. To remove any particulate debris, samples were spun down at 10,000 x g at 4°C for 10 min, and the supernatant was placed in a fresh tube. Protein was quantitated using a 1:5 dilution of the sample using the EZQ assay (Thermo Fisher Scientific, Waltham, MA). Protein used for subsequent mass spectrometry was standardized across biological replicates (16 µg).

Transferred Ejaculate Isolation

To identify SFPs that are unequivocally transferred to the female, we used a reverse-labeling technique pioneered in *Drosophila* by Findlay et al. (36) and adapted to *Ae. aegypti* by Sirot et al. (27). Females labeled with ^{15}N were mated to unlabeled males, allowing the identification of only transferred ejaculate proteins via MS/MS. -

Males were reared as described above. As larvae, females were labeled with ^{15}N using the rearing methodology of Sirot *et al.* (27). Briefly, a prototrophic yeast strain (D273-10B) was grown in media whose only nitrogen source was ^{15}N ammonium sulfate (Cambridge Isotope Laboratories; Cambridge, MA). Yeast were grown to saturation, pelleted, and resuspended in PBS to a final volume of one sixteenth of the growth media. A few drops of yeast slurry were provided to newly hatched first instar larvae after vacuum hatching. One day after hatching, 200 larvae were placed in a rearing tray with 200 mL water from a previous cohort of ^{15}N-yeast-reared mosquitoes (to seed their rearing environment with beneficial microbiota) and 800 mL of deionized water. Larvae were fed 4 mL of labeled yeast slurry a day after hatching, and again at 4 d after hatching. Pupae were isolated into individual tubes as described above, and females were put into an 8 L bucket cage and provided sucrose solution *ad libitum*. Sucrose was replaced every 2 d to preclude the introduction of unlabeled nitrogen via microbial contamination.

At 4 - 5 dpe, matings between labeled females and unlabeled males were observed as in Degner and Harrington (35). Because mosquito seminal fluid is known to contain proteases (27), mosquitoes were dissected in saline with protease inhibitors (cOmplete Mini Protease Inhibitor Cocktail; Sigma Aldrich, St. Louis, MO). In contrast to Sirot *et al.* (27), we dissected only bursae (and not spermathecae). Adjoining tissue associated with the gonotreme (vaginal lips) was left in place and acted as a tissue barrier that prevented escape of seminal fluid into the surrounding saline. We cannot rule out the possibility that some seminal proteins may be processed, modified, or trafficked outside of the bursa in the short time between

mating and dissection. To minimize these processes, we immediately placed females on ice after mating and dissected them within 3 min of mating. Upon excision, bursae (n = 35 - 43 per replicate) were transferred to 33 µL 1x Laemmli buffer diluted from a 2x stock solution with saline, with 2.5% β-mercaptoethanol and 1x protease inhibitors. Samples were sonicated on high with a Bioruptor UCD-200 (Diagenode, Liège, Belgium) for 30 s, boiled for 10 min, sonicated for 30 s, and centrifuged at 10,000 x g at 4°C for 10 min. The supernatant was removed; 30 µL were frozen at -80°C, and 2 µL were diluted 1:3 with buffer for protein quantitation using the EZQ assay (Thermo Fisher Scientific, Waltham, MA). In parallel, we also prepared samples of bursae from virgin, labeled females from each cohort to assess the efficiency of ^{15}N-labeling. Mated and virgin bursae samples contained 18 and 8 µg of protein, respectively.

Tandem mass spectrometry analysis

Solubilized proteins were separated on a 1-dimensional SDS-PAGE gel and split into 6 fractions, with two biological replicates run in parallel (Figure 5.1). Gel fractions were cut into 1 mm cubes, washed, and dehydrated (37). Subsequently, proteins were reduced with dithiothreitol and alkylated with iodoacetamide (38). Gel pieces were subsequently digested in 50 µL trypsin in 50 mM ammonium bicarbonate, 10% acetonitrile (20 ng/µL) at 37°C for 16 h. Resultant peptides were extracted with two washes of 50% acetonitrile, 5% formic acid, and one with 90% acetonitrile, 5% formic acid. Extracts from each wash were pooled, lyophilized, reconstituted in 0.5% formic acid, and subjected to nanoLC-ESI-MS/MS analysis

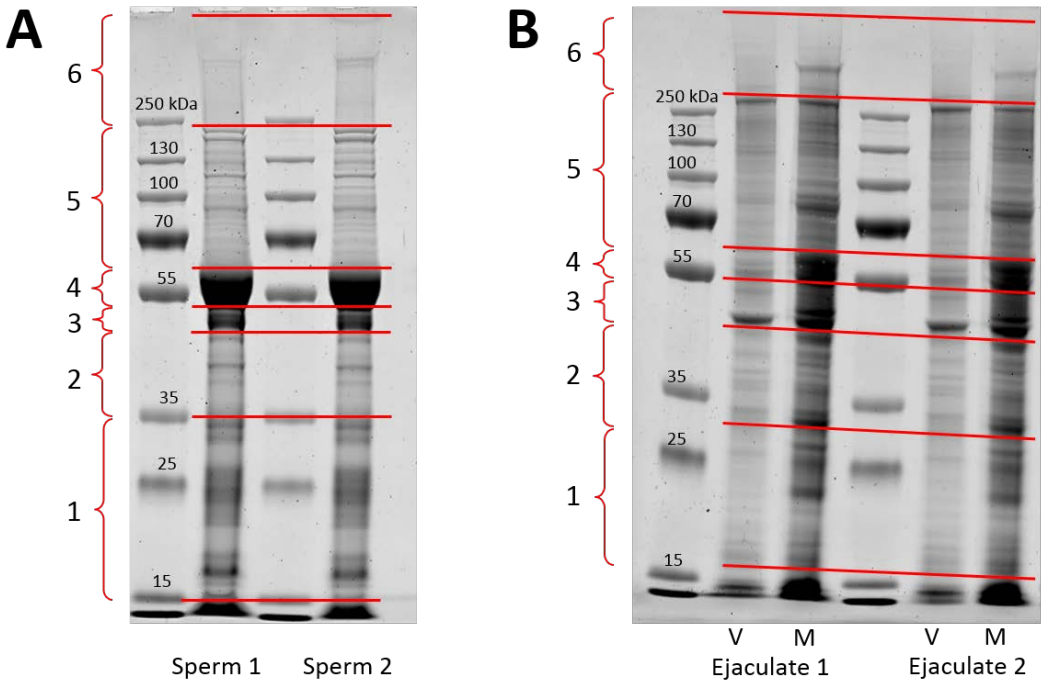


Figure 5.1. One-dimension SDS-PAGE gel separation of samples. Sperm (A) and ejaculate (B) protein fractions (1 - 6) analyzed by LC-MS/MS. Biological replicates were run in parallel on 10% bis-tris SDS-PAGE gel with MOPS running buffer and stained with colloidal Coomassie. V, virgin bursae; M, mated bursae. Labeling efficiency of females was verified through the analysis of fraction 5 from virgin females.

using an Orbitrap Fusion Tribrid mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with nanospray Flex Ion Source, and coupled with a Dionex UltiMate 3000RSLC nano system (Thermo, Sunnyvale, CA). Peptide samples were injected onto a PepMap C-18 RP nano trap column (5 µm, 100 µm i.d. x 20 mm, Dionex) with nanoViper fittings at 20 µL/min flow rate for desalting. Samples were then separated on a PepMap C-18 RP nano column (2 µm, 75 µm x 15 cm) at 35°C, followed by elution on a 90 min gradient of 5% to 35% acetonitrile in 0.1% formic acid at 300 nL/min. Finally, a 5 min ramping to 90% acetonitrile in 0.1% formic acid and a 5 min hold at this eluent completed each run cycle. Between cycles, the column

was re-equilibrated for 25 min using 0.1% formic acid. The Orbitrap Fusion was run in positive spray ion mode with spray voltage set at 1.6 kV and a source temperature at 275°C. External calibration for FT, IT, and quadrupole mass analyzers was performed. In data-dependent acquisition analysis, the instrument was operated using FT mass analyzer in MS scan to select precursor ions followed by 3 s "Top Speed" data-dependent CID ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10,000 and normalized collision energy of 30%. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 375-1575. Dynamic exclusion parameters were set at repeat count 1 with a 20 s repeat duration, an exclusion list size of 500, and 40 s of exclusion duration with ±10 ppm exclusion mass width. The activation time was 10 ms for CID analysis. All data were acquired under Xcalibur 3.0 operation software (Thermo-Fisher Scientific). All post-quantitation sample preparation was conducted at the Cornell Biotechnology Resource Center.

Peptide identification and protein annotation

RAW data were converted to mzML format using msconvert from the Trans-Proteomic Pipeline (TPP v5.0 (Typhoon) rev 0; (39)), using the default peak filtering parameters. The processed data from each MS/MS run was analyzed by X!Tandem (40) and Comet (41) against the *Ae. aegypti* L5.0 protein database (GCF_002204515.2; 42). Only the longest protein isoform of each gene was included in the search database, resulting in a database of 14,626 proteins. For X!Tandem, a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 10.0 PPM were

used. For Comet, a fragment bin tolerance of 1.0005 with a 0.4 offset and a parent ion tolerance of 10.0 PPM were used. Iodoacetamide derivative of cysteine was specified as a fixed modification, whereas oxidation of methionine and deamidation of glutamine and asparagine were specified as variable modifications. Peptides were allowed up to two missed trypsin cleavage sites. All downstream analyses were conducted using the Trans-Proteomic Pipeline (TPP v5.0 (Typhoon) rev 0; 39). False Discovery Rates (FDRs) for each tissue (sperm or ejaculate) were estimated with a randomized decoy database using PeptideProphet (43), employing accurate mass binning model and the nonparametric negative distribution model. X!Tandem and Comet PeptideProphet results were merged using iProphet (44), to provide more robust peptide identification. Peptide identifications were accepted if they could be established at greater than 95.0% iProphet probability, and protein assignations were accepted if they could be established at greater than 99.0% probability. Proteins that contained identical peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy parsimony principles. Proteins were required to be identified by two or more unique peptide spectral matches to be included.

Verification of labeling efficiency

For ejaculate samples, protein from virgin female controls was run on the same gel as protein from their mated counterparts, and labeling efficiency was verified on a representative fraction in each cohort's virgin sample (Figure 5.1). No peptides or proteins were identified using our statistical criteria in the labeled virgin female samples when searched using standard, unlabeled mass parameters. Thus,

whole female labeling was complete in relation to MS/MS peptide and protein identification and precluded the identification of female proteins from mated female bursae.

Protein quantitation

Protein quantitation was conducted using the semi-quantitative spectral counting approach implemented by the APEX Quantitative Proteomics Tool (45). The 50 proteins with the highest protein identification probabilities (as determined by iProphet) were utilized as the training dataset. The 35 physicochemical properties available in the APEX tool were used for prediction of peptide detection/non-detection in the construction of a training dataset file. Protein probabilities (O_i) were computed using the Random Forest classifier algorithm trained with the data set generated in the previous step. APEX protein abundances per sample were calculated using the protXML file generated by ProteinProphet.

Experimental design and statistical rationale

To ensure the reproducibility of protein identifications, two biological replicates of each tissue were analyzed, with each replicate prepared from independent cohorts of mosquitoes. The reproducibility of protein identification was high for both tissues (see results). We therefore combined both replicates to increase sensitivity and proteome coverage (see above). To control for the possibility that unlabeled female-derived proteins were identified in our ejaculate samples, we assessed labeling efficiency by conducting mass spectrometry on virgin bursae alone,

using a representative gel slice from each biological replicate (Figure 5.1). False Discovery Rates (FDRs) were estimated with a randomized decoy database using PeptideProphet (43), employing accurate mass binning model and the nonparametric negative distribution model. For differential mRNA expression, GO, and KEGG pathway analyses, FDR correction was performed by applying the Benjamini-Hochberg method on the calculated *p*-values (46).

Transcriptome analysis of testes and male accessory glands

Testes were harvested from males at 1 dpe and transferred to TRIzol. Because mature sperm are actively produced at this age (47), and spermatogenesis is at its peak (48, 49), testes at this age likely contain the majority of transcripts that contribute to the testicular sperm proteome. Male accessory glands (MAG) including the connecting ejaculatory duct were dissected from virgin males aged 6 and 8 dpe, and care was taken to remove as much of the adjoining seminal vesicle as possible with a minutien pin. We also analyzed MAG from mated males at the same age. Previous work has demonstrated that *Ae. aegypti* males become depleted after mating with three to five females in succession, and seminal fluid is slowly regenerated over 48 h (50). In our study, we provided males with four virgin females for a period of 8 h (beginning 2 h after simulated dawn) to allow for male seminal fluid depletion. On average, each male mated with more than three females in this period (as determined by dissection of females' spermathecae). Males were dissected in saline 16 h after their mates had been removed. We generated four biological replicates from independent cohorts for each treatment, and each replicate contained combined tissue

from 20-40 (testes) or 40-60 (MAG) males. Total RNA was extracted from each sample in Trizol following manufacturer's instructions (Invitrogen, Carlsbad, CA). Poly-A mRNA was isolated and cDNA libraries were prepared using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Amplified cDNA products were run on an AATI Fragment Analyzer (Advanced Analytical Technologies, Inc.; Hialeah, FL) so that the cDNA was of sufficient concentration for sequencing. Library concentrations were balanced using digital PCR (51), and each of the 12 uniquely barcoded samples were sequenced in one lane using the Illumina HiSeq 2500 platform with 100 bp reads. All sequencing was conducted at the Cornell Biotechnology Resources Center.

For further analysis of the transcriptomes, we included additional, publicly available data to evaluate tissue-biased gene expression. These include gonadectomized male carcass (SRP075464; 52) and a virgin female reproductive tract sample (SRP068996; 53). Raw RNA-seq reads were processed by trimming the first 10 bases from the 3' position, followed by quality trimming of both ends to a minimum quality Phred score of 20 (Sickle v.1.210; 54). Processed reads were then mapped to the *Ae. aegypti* genome (VectorBase release L5.1; 42) using Hisat2 (v.2.1.0; 55) with default parameters, and transcript abundance was estimated for each sample with StringTie (v.1.3.4; 56). Raw counts for each sample were extracted from the StringTie abundance estimates using the auxiliary "prepDE.py" script provided on the StringTie website (https://ccb.jhu.edu/software/stringtie/). Signal peptides in the translated transcriptome were predicted *in silico* using a local installation of SignalP (v.4.1; 57).

We used raw counts from the RNA-seq samples to (**1**) classify genes based on tissue-biased expression in the MAG and testes, and (**2**) identify genes differentially expressed between virgin and mated MAG (based on transcripts per million; TPM). Count matrices were filtered to remove low abundance transcripts (counts per million < 5). First, we compared expression levels of testes or virgin MAG (in the present study) to levels in three other tissues: gonadectomized male carcass (52), virgin female reproductive tract (53), and virgin MAG (in comparison with testes) or testes (in comparison with virgin MAG). We classified genes as testes- or MAG-biased if they had >2-fold higher transcript abundance compared to other samples at a minimum FDR cutoff of 0.05 (edgeR v.3.23.2; 58). We identified differentially expressed genes between virgin and mated MAGs as having >2-fold abundance difference at an FDR cutoff of 0.01. Using the same differential expression criteria, we also re-analyzed data from female reproductive tracts of virgin and just-mated females (53) to identify transcripts putatively transferred to females in the ejaculate using current annotated gene models. Finally, we assessed tissue-biased mRNA expression of putative SFPs and sperm proteins using a Wilcoxon signed rank test with continuity correction to test whether the mean log2 ratio of MAG/testis mRNA expression significantly deviated from zero.

Chromosomal distribution of male reproductive genes

To evaluate the chromosomal distribution of SFPs, sperm proteins and MAG/testes-biased genes, we calculated the expected number of genes for each class on each chromosome, assuming a random distribution of genes across the genome.

We then multiplied the total number of genes within each class by the expected proportion for each chromosome (based on the proportion of total genes on that chromosome) to establish an observed/expected ratio. We also calculated this ratio for a 123 Mb region on chromosome 1 that surrounds the sex determining locus and has low rates of recombination (59). A Chi-square test ($df = 1$) for each gene class per chromosome was used to test for biased representation.

Orthology relationships and functional enrichment analysis

Protein orthology was assessed using a local installation of OrthoDB with default SWIFT and clustering parameters (56). Briefly, best reciprocal hits (BRHs) were first identified using an all-vs-all approach via the algorithm SWIPE (60), and clusters were built progressively with e-value cutoffs of 1E-3 for triangulating BRHs and 1E-6 for pair-only BRHs. One-to-one, one-to-many and many-to-many relationships were included in subsequent analyses. Protein sequences for *Ae. aegypti* and *Ae. albopictus* were retrieved from NCBI (GCF_002204515.2 and GCF_001876365.2, respectively), and *Drosophila melanogaster* protein sequences were retrieved from FlyBase (r6.18). Protein sequences for each species were filtered to retain only the longest isoform for each gene. Sperm proteins and SFPs for *Ae. albopictus* and *D. melanogaster* were based on previous mass spectrometry-based proteomic studies (28, 32, 36). Because SFP identifications by Boes *et al.* (28) were made using a *de novo* transcriptome (the *Ae. albopictus* genome had not yet been released), we converted the original SFP identifications to their current accession numbers by BLASTing assembled transcripts from Boes *et al.* (28) to the assembled

Ae. albopictus genome (61). All Gene Ontology (GO) analyses were conducted using GOseq (62), with gene lengths derived from the longest transcript of each assembled StringTie gene. GO terms were extracted from BLAST results against the SwissProt database (www.uniprot.org). KEGG pathway analysis was performed with clusterProfiler (63) using the *Ae. aegypti* KEGG database.

Results

Sperm and ejaculate proteome characterization

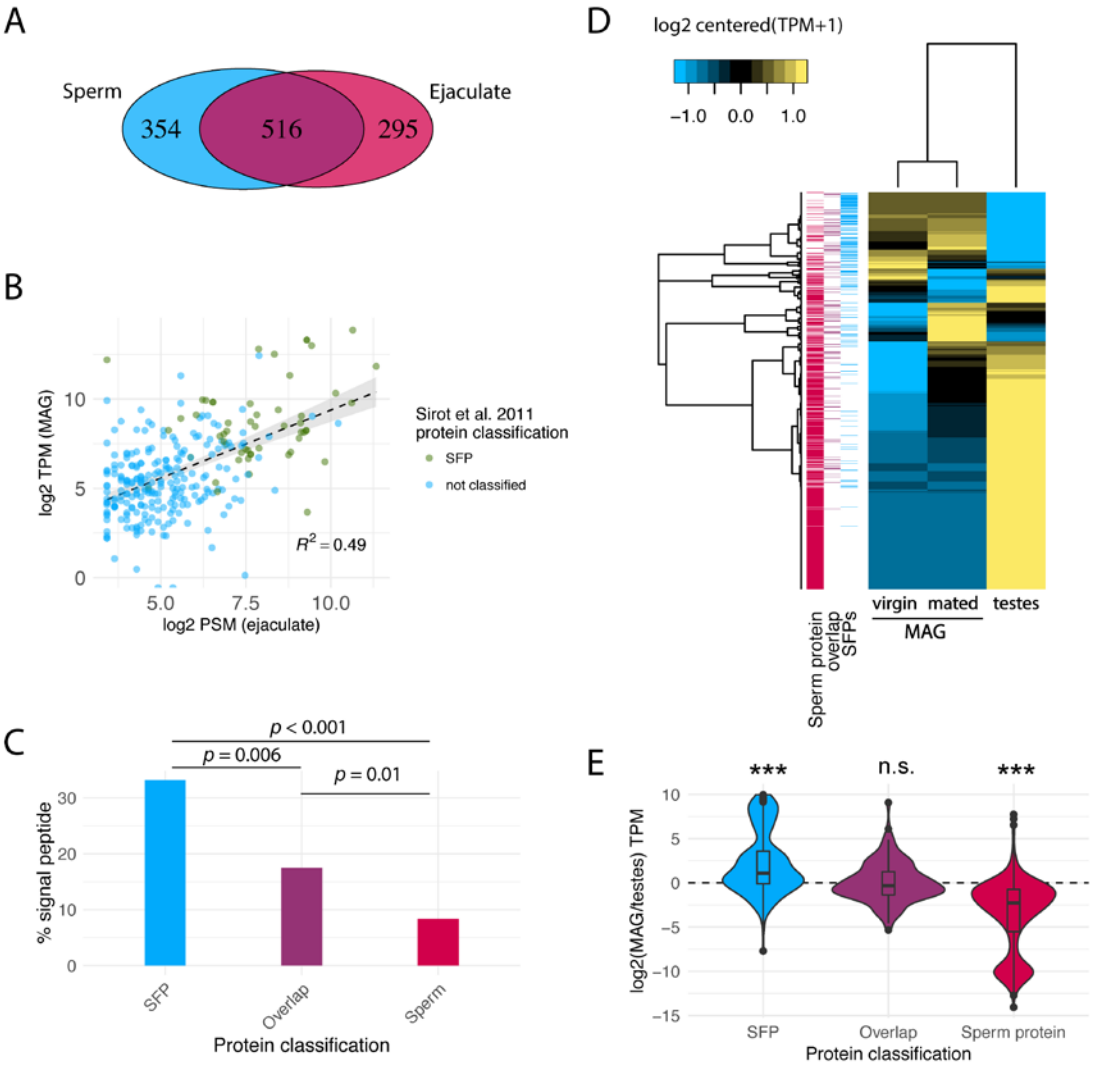
To identify *Ae. aegypti* proteins transferred by males to females during mating and to distinguish sperm and non-sperm seminal fluid components, we used MS/MS to analyze proteins from (**1**) purified sperm isolated from male seminal vesicles and (**2**) whole ejaculate from bursae of mated females. For our analysis of the whole ejaculate, females were labeled with heavy nitrogen to preclude detection of female-contributed proteins. Labeling efficiency was determined to be complete with regard to peptide and protein identification (see Experimental Procedures). Two replicates were analyzed per sample. High levels of reproducibility were observed, including 85% of sperm proteins and 78% of ejaculate proteins identified in both biological replicates. Similar levels of inter-replicate consistency have been reported for sperm proteomes of other organisms (34). To maximize protein identification, we combined spectra across biological replicates for our final proteome determination, resulting in 54,894 peptide spectral matches (PSMs) for sperm, and 30,801 for the ejaculate

(Table S1[*]). The nearly two-fold disparity in PSMs is due to the contribution of labeled female proteins in the ejaculate sample. In total, 870 and 811 proteins with at least 2 unique PSMs were identified in sperm and ejaculate, respectively (Table S2). Sperm proteins were identified by an average of 11.4 unique PSMs and 62.8 total PSMs per protein. Ejaculate proteins were identified with an average of 8.0 unique PSMs and 37.8 total PSMs. As was expected given the substantial contribution of sperm cells to ejaculate composition, extensive overlap was observed between sperm and ejaculate proteomes; 516 proteins were detected in both samples, while 354 proteins were only identified in sperm and 295 proteins were uniquely detected in the ejaculate (Figure 5.2A).

The primary goal of this study was to use MS/MS with higher sensitivity and accuracy to expand upon the prior characterization of *Ae. aegypti* SFPs and sperm by Sirot *et al.* (27). They identified 74 SFPs that mapped to the recently refined *Ae. aegypti* genome (42); some of the 93 SFPs described by Sirot *et al.* (27) do not map to the new genome or are now part of larger, fused gene models. Of these, we identified 60 (81%) in our ejaculate sample, 32 of which were also identified in our purified sperm sample. It is noteworthy that we detected an additional 5 SFPs from Sirot *et al*. (27), but these were not included as SFPs because they did not meet our two unique peptide inclusion criteria. As such our proteomic characterization expands the previous *Ae. aegypti* SFP characterization.

[*] All supplementary tables can be accessed at https://www.mcponline.org/content/early/2018/12/14/mcp.RA118.001067/tab-figures-data

Figure 5.2. Characteristics of the *Ae. aegypti* sperm and ejaculate proteomes. (A) Venn diagram representing the number of proteins identified by ≥2 unique peptides using LC-MS/MS in sperm (red) and ejaculate (blue) proteomes. (B) Scatter plot of the ejaculate protein (log_2PSM) and virgin MAG mRNA (log_2TPM) abundances of the 177 high-confidence SFPs and 103 sperm/SFP overlap proteins. Proteins classified as SFPs in the Sirot *et al*. study are highlighted in green and those not identified in Sirot *et al*. (27) are highlighted in blue. Dotted line is a linear model fit, and gray shading represents the 95% confidence interval. (C) Percentage of proteins containing a predicted signal peptide sequence among SFPs, sperm, and sperm/SFP overlap protein classes; all proportions are significantly different from each other (Chi-square, $p < 0.05$). (D) Heatmap representing the mRNA abundance profile of male reproductive classes identified in this study. Cladograms on the left and top represent Pearson cluster grouping of genes and samples, respectively. Annotation bars on the left indicate the protein classification for each gene. (E) Violin plots displaying the fold-change (log_2) distribution of mRNA abundance between MAG and testes samples for each male reproductive protein class. Width of violins represents the number of proteins at any given TPM ratio; boxes represent inner quartiles and outliers drawn using the Tukey method. Asterisks indicate groups that are significantly different from each other (Wilcoxon Signed rank test; *** $p < 0.001$).


A
Sperm
Ejaculate
354
516
295
B
log2 TPM (MAG)
log2 PSM (ejaculate)
$R^2 = 0.49$
Sirot et al. 2011
protein classification
SFP
not classified
C
$p < 0.001$
$p = 0.006$
$p = 0.01$
% signal peptide
SFP
Overlap
Sperm
Protein classification
D
log2 centered(TPM+1)
−1.0 0.0 1.0
virgin mated testes
MAG
Sperm protein
overlap
SFPs
E
***
n.s.
***
log2(MAG/testes) TPM
SFP
Overlap
Sperm protein
Protein classification

Refined seminal fluid protein classification

Because the ejaculate is a complex mixture of sperm and seminal fluid, we applied stringent inclusion criteria to our ejaculate samples to refine the delineation between SFPs and sperm proteins. We first removed 10 proteins involved in protein translation (i.e. ribosomal proteins, translation initiation factors, and elongation factors; Table S2) from the list of putative SFPs. These proteins exhibit ubiquitous patterns of expression, including both MAG and testes, and are unlikely to be *bona fide* secreted SFPs. Although we cannot rule out that they are secreted SFPs, their presence in our samples may also be the result of holocrine secretion in the accessory glands, inclusion in apocrine vesicles (64, 65), or transfer of MAG cells to the female, as has been described in *D. melanogaster* (66). To reduce the possible inclusion of sperm proteins that were absent in our sperm proteome but present in the ejaculate (perhaps due to low abundance), we define "high confidence SFPs" as proteins with a minimum of 6 total PSMs in the ejaculate, 2 unique PSMs, and not present in our sperm proteome; this resulted in 177 high-confidence SFPs (Table S2).

Previous analyses of insect sperm proteomes have consistently identified proteins generally considered to be SFPs (*i.e.* highly expressed in the MAG and believed to be secreted molecules transferred to females as non-sperm components (32, 67, 68)). To identify proteins predominantly produced by the MAG, but identified in both our sperm and ejaculate sample, we used a 2.5-fold greater protein abundance threshold in the ejaculate relative to sperm. This resulted in the identification of 103 additional putative SFPs, which we label as "sperm/SFP overlap" (Table S2), including 53 with 5-fold greater protein abundance in the

ejaculate relative to sperm. In total, this resulted in a combined SFP proteome of 280 proteins.

To better understand the relationship between SFPs identified in the current study and by Sirot *et al.* (27), we examined protein abundance and transcript levels of both protein sets (50 of the 74 SFPs identified by those authors are present in our SFP proteome). This revealed that SFPs identified by Sirot *et al.* (27) were significantly more abundant than the remainder of our 280 SFPs (Figure 5.2B; Wilcoxon rank sum test; $W = 3786$, $p < 0.001$), consistent with the higher sensitivity and coverage of the MS/MS methods utilized in this study. Proteins identified by Sirot *et al.* (27) also exhibited significantly higher levels of MAG expression relative to SFPs identified in this study (Figure 5.2B; Wilcoxon rank sum test; $W = 3005$, $p < 0.001$). We therefore conclude that the sensitivity of our SFP characterization resulted in the addition of a greater number of low abundance SFPs.

To evaluate our SFP characterization, we explored three types of analyses. First, we determined the presence of predicted signal peptides in identified proteins — a hallmark of secreted proteins. Among our high-confidence SFPs, ~33% contained signal peptides, in comparison to only ~9% of sperm proteins (Chi-square = 69.2, $p < 0.001$). Additionally, ~17% of the sperm/SFP overlap proteins had a predicted signal peptide — also significantly more than the sperm proteome (Chi-square = 6.47, $p = 0.01$), and less than high-confidence SFPs (Chi-square = 7.7, $p = 0.006$; Figure 5.2C). Second, we used RNA-seq to characterize the transcriptomes of the MAG and testis, the predominant source tissues of proteins in our analysis. As expected, SFPs (Wilcoxon Signed rank test; $p < 0.001$) and sperm proteins (Wilcoxon

Signed rank test; $p < 0.001$) exhibit MAG- and testis-biased expression, respectively, while the sperm/SFP overlap proteins exhibit less tissue-biased expression overall (Wilcoxon Signed rank test; $p = 0.36$; Figures 5.2D-E). Fewer than 1% of all identified proteins were not present in the transcriptome of their predicted source tissue (9 sperm proteins, 2 high-confidence SFPs, and 1 sperm/SFP overlap protein). Third, we assessed the amount of high-confidence SFP protein abundance variation explained by variation in MAG expression levels. This revealed a significant correlation between protein and transcript abundance ($R^2 = 0.49$, $F = 88.24$, $p < 0.001$; Figure 5.2B). In conjunction, these results support our proteomic characterization of SFPs produced in the MAG, including proteins present in both sperm and ejaculate samples but highly enriched in the ejaculate proteome.

Functional enrichment in sperm and seminal fluid proteomes

Gene ontology (GO) categories enriched in the *Ae. aegypti* sperm proteome are largely similar to those found in other insects' sperm (Table 5.1, Table S3; 32, 34, 67, 68). Proteins associated with mitochondria and the axoneme were the most enriched cellular components in the sperm proteome. Proteasome components were also enriched. Over-represented biological processes in sperm include nucleotide biosynthesis, metabolic processes related to the tricarboxylic acid cycle, and proteins regulating ciliar function. Nucleotide binding, ion transport, and oxidoreductase activity were enriched molecular functions in sperm. KEGG pathways enriched in the sperm proteome were dominated by those involved in metabolism, including carbon metabolism (e.g., pyruvate and butyrate metabolism, the TCA cycle, and oxidative

Table 5.1. Gene Ontology analysis of sperm proteins, high-confidence SFPs, and sperm/SFP overlap. List of significant terms is abbreviated to exclude redundancy and to focus on terms discussed in text. For exhaustive list, see Table S3. FDR; false discovery rate.

Dataset	Ontology	GO Term	Proteins (total in category)	FDR
Sperm Proteome	Biological Process	organonitrogen compound metabolic process	102 (978)	5.30E-11
		carboxylic acid metabolic process	100 (553)	1.90E-23
		electron transport chain	13 (55)	6.50E-05
	Cellular Component	mitochondrion	150 (834)	1.20E-45
		microtubule	36 (210)	1.30E-07
		cilium	32 (154)	1.70E-08
		proteasome complex	31 (47)	2.10E-25
		dynein complex	19 (40)	1.70E-11
	Molecular Function	nucleoside phosphate binding	187 (1629)	5.70E-20
		hydrolase activity	163 (2153)	4.70E-04
		oxidoreductase activity	103 (839)	2.00E-13
		threonine-type endopeptidase activity	14 (15)	8.30E-16
High-confidence SFP proteome	Biological Process	organonitrogen compound metabolic process	30 (978)	0.009
		carboxylic acid metabolic process	21 (553)	0.033
		hexose metabolic process	10 (79)	7.50E-04
	Cellular Component	extracellular region part	33 (1116)	0.029
	Molecular Function	hydrolase activity	62 (2153)	0
		nucleoside phosphate binding	43 (1629)	0.04
		peptidase activity	27 (739)	0.006
Sperm/ SFP overlap	Biological Process	carboxylic acid metabolic process	29 (553)	7.20E-11
		vesicle-mediated transport	15 (536)	0.017
		protein folding	10 (113)	2.60E-05
		ATP hydrolysis coupled proton transport	8 (25)	4.30E-08
		oocyte microtubule cytoskeleton polarization	3 (10)	0.014
	Molecular Function	nucleoside phosphate binding	39 (1629)	3.40E-06

phosphorylation) as well as the metabolism of several classes of amino acids (Table S4).

In our high-confidence SFP proteome, extracellular structure was significantly enriched amongst cellular component categories, further supporting the accuracy of our SFP identification. Over-represented biological processes include proteolysis and both carbohydrate and amide metabolism. Significantly enriched molecular functions include hydrolase activity and peptidase activity. This observation is consistent with the widespread presence of proteolytic enzymes and regulators in SFPs of other insects (reviewed in 69). The sperm/SFP overlap proteome shared several enriched categories with both the sperm and high-confidence SFP proteomes, including carboxylic acid metabolic processes and nucleotide binding. Several additional GO terms emerged in this protein set as well, including ATP hydrolysis-coupled proton transport, vesicle-mediated transport, and protein folding, the latter of which was previously reported by Sirot et al. ((70); Table 5.1, Table S3). Enriched KEGG groups in the combined seminal fluid (high-confidence SFP and sperm/SFP overlap) proteomes included those related to phagosomes and lysosomes, as well as pathways related to carbon metabolism and gluconeogenesis.

Orthology with sperm proteins and SFPs in other species

We next examined orthology of sperm proteins and SFPs in two different species: *D. melanogaster*, given its well-characterized sperm proteome and SFPs (32, 36), and *Ae. albopictus*, which is the closest species to *Ae. aegypti* with characterized SFPs (28). Orthology was determined between the complete genome of all three

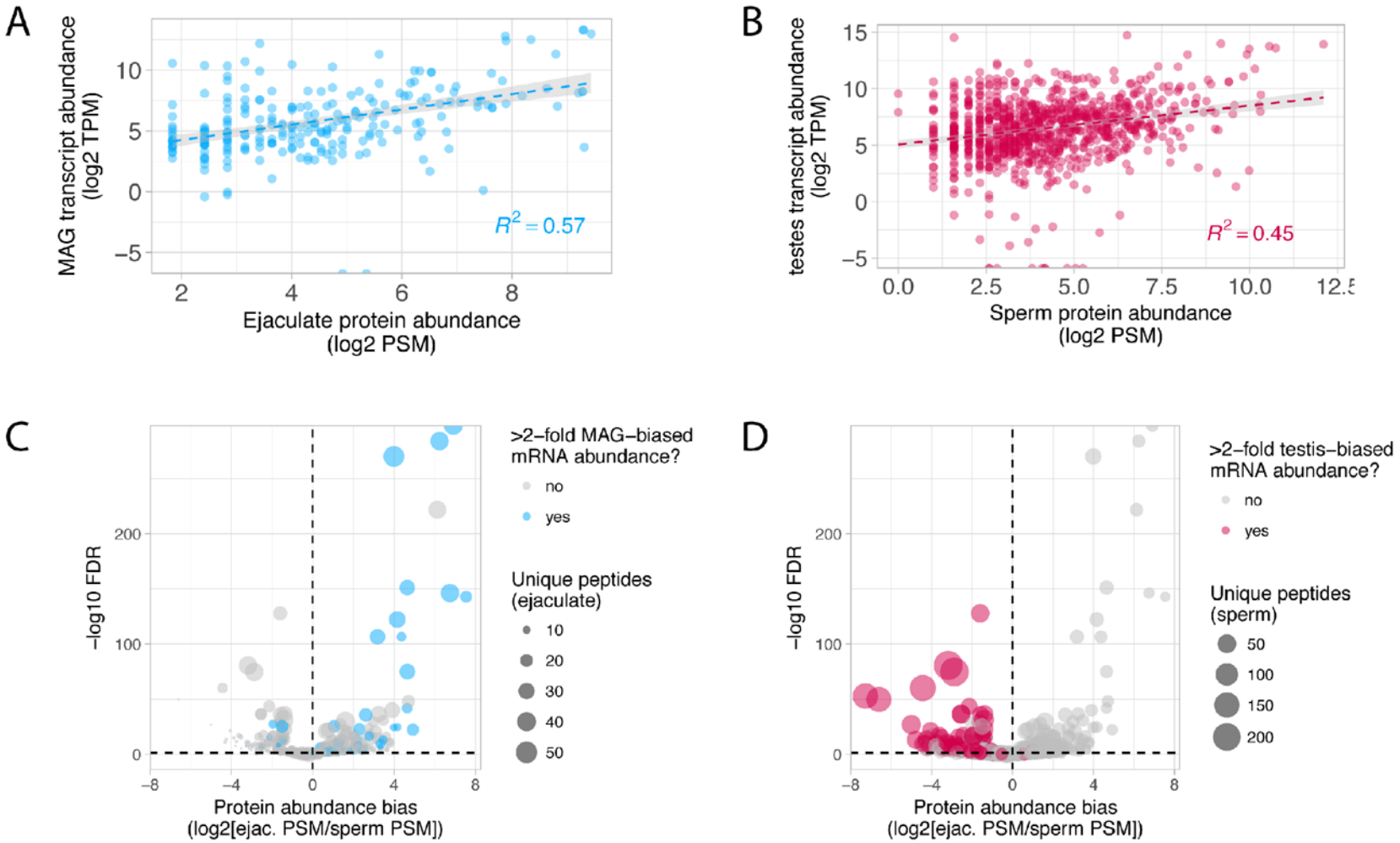
species, and then orthologs of *Ae. aegypti* SFPs and sperm proteins also classified as SFPs or sperm proteins in the other species were identified. We focused solely on SFPs in the comparison with *Ae. albopictus,* because a thorough sperm proteome is lacking for this species.

Overall, ~87% and ~98% of proteins in the *Ae. aegypti* genome have an ortholog (either as one-to-one or one-to-many relationships) in *D. melanogaster* and *Ae. albopictus*, respectively. Among proteins unique to the *Ae. aegypti* sperm proteome, 760 (99%) have an ortholog in *D. melanogaster*, and 451 (59%) of these are also found in the *D. melanogaster* sperm proteome (Table S2; 32, 68). Out of the 280 *Ae. aegypti* SFPs characterized in our study (including 177 high-confidence and 103 sperm/SFP overlap proteins), 275 (98%) have orthologs in *D. melanogaster*. Of these, only 11 have also been identified as *D. melanogaster* SFPs (4%; Table S2). Proteins that contribute to the seminal fluid proteome have therefore diverged extensively during Dipteran evolution. Orthologs were identified in the *Ae. albopictus* genome for 275 (98%) of our SFPs. Of the *Ae. albopictus* SFPs identified to date (28), 86 (43%) were classified as SFPs in our study (Table S2).

MAG and testis transcriptome characterization and differential expression

We used short-read RNA sequencing to examine gene expression in testes and MAGs to (**1**) identify transcripts with tissue-biased expression, (**2**) compare transcript and protein abundance, (**3**) assess the transfer of male RNAs to females during mating and (**4**) characterize the effect of mating on MAG gene expression. In both MAG and testes, we found a significant correlation between transcript abundance and

Figure 5.3. (A) and (B) Scatterplots of normalized mRNA and protein abundance in MAG/ejaculate (A) and testes/sperm (B). The line and gray shading represent a linear model fit with its 95% confidence interval respectively. Coefficients of determination (R^2) are indicated. (C) and (D) Volcano plots of protein abundance differences for all proteins detected in both the ejaculate and sperm samples. Proteins that show >2-fold mRNA expression-bias in MAG or testes tissue are indicated in blue (MAG-biased) or red (testes-biased), and the size of each point corresponds to the number of unique peptides detected for each protein.


A
MAG transcript abundance (log2 TPM)
Ejaculate protein abundance (log2 PSM)
$R^2 = 0.57$
B
testes transcript abundance (log2 TPM)
Sperm protein abundance (log2 PSM)
$R^2 = 0.45$
C
−log10 FDR
Protein abundance bias (log2[ejac. PSM/sperm PSM])
>2−fold MAG−biased mRNA abundance?
no
yes
Unique peptides (ejaculate)
10
20
30
40
50
D
−log10 FDR
Protein abundance bias (log2[ejac. PSM/sperm PSM])
>2−fold testis−biased mRNA abundance?
no
yes
Unique peptides (sperm)
50
100
150
200

protein abundance for high confidence SFPs and sperm proteins (SFPs: R^2= 0.57, F = 110.4, $p < 0.001$; sperm: R^2 = 0.45, F = 223.8, $p < 0.001$; Figures 5.3A-B). As such, variation in transcript abundance explains a substantial amount of protein variation in both of our samples. In total, ~11,000 and ~7,000 genes had detectable mRNA expression in the testes and MAG, respectively. However, only a subset of these exhibit >2-fold expression bias in testes or MAG compared to other tissues (testes: 2,863; MAGs: 1,485). We examined the association between tissue-biased mRNA expression and differential protein abundance for proteins that were detected in both ejaculate and sperm samples. These results demonstrate that proteins with significant protein abundance differences between sperm and ejaculate samples also tend to show >2-fold tissue-biased mRNA expression (Figures 5.3C-D), further supporting our SFP classification criteria (see above). However, we note that this relationship is less faithful for lower abundance proteins.

Males regenerate seminal fluid over the course of 48 h after depleting their reserves by repeated insemination (50). While transcripts that are upregulated or abundant during this period of replenishment should not be assumed to be SFPs, we nonetheless reasoned that MAG transcriptional regulation after mating might inform our understanding of pathways required to produce depleted SFPs. Differential expression analysis of virgin and mated MAGs' transcriptomes revealed a significant bias towards gene upregulation in mated males, with 320 transcripts that are upregulated and 126 that are downregulated after mating (binomial test; $p < 0.001$; Figure 5.4A). In contrast to downregulated transcripts—which were not enriched for any functional category—upregulated transcripts were enriched for several GO

Table 5.2. Gene Ontology analysis of upregulated genes in MAGs after mating.

Ontology	GO Term	Proteins (total in category)	FDR
Biological Process	protein targeting to ER	9 (17)	1.88E-07
	ubiquitin-dependent protein catabolic process	21 (197)	5.70E-06
	Response to stress	56 (1321)	8.11E-04
	cellular amino acid metabolic process	19 (246)	0.001
	signal peptide processing	4 (7)	0.004
Molecular Function	aminoacyl-tRNA ligase activity	12 (52)	2.46E-06
	threonine-type endopeptidase activity	7 (15)	1.36E-05
Cellular Component	proteasome complex	17 (47)	1.18E-12
	endoplasmic reticulum part	33 (660)	0.005
	small nucleolar ribonucleoprotein complex	5 (18)	0.015
	organelle inner membrane	18 (288)	0.031
	nucleolus	20 (345)	0.035

categories, many of which are consistent with this tissue's primary function of producing secreted proteins (Table 5.2). For example, amino acid metabolism and aminoacyl-tRNA ligase activity were found to be enriched. Enrichment of proteins involved in ubiquitin-dependent protein catabolism and those making up components of the proteasome suggests that there may be extensive protein recycling as seminal fluid is regenerated. Furthermore, the over-representation of proteins associated with endoplasmic reticulum targeting and signal peptide processing suggests that many seminal proteins are targeted for post-translational processing and export from the cell. We also note that a variety of immune-related genes are upregulated in MAGs after mating, including a family of *Defensin* antimicrobial genes (Figure 5.4A). Lastly, a similar pattern was observed among our characterized SFPs, of which 19

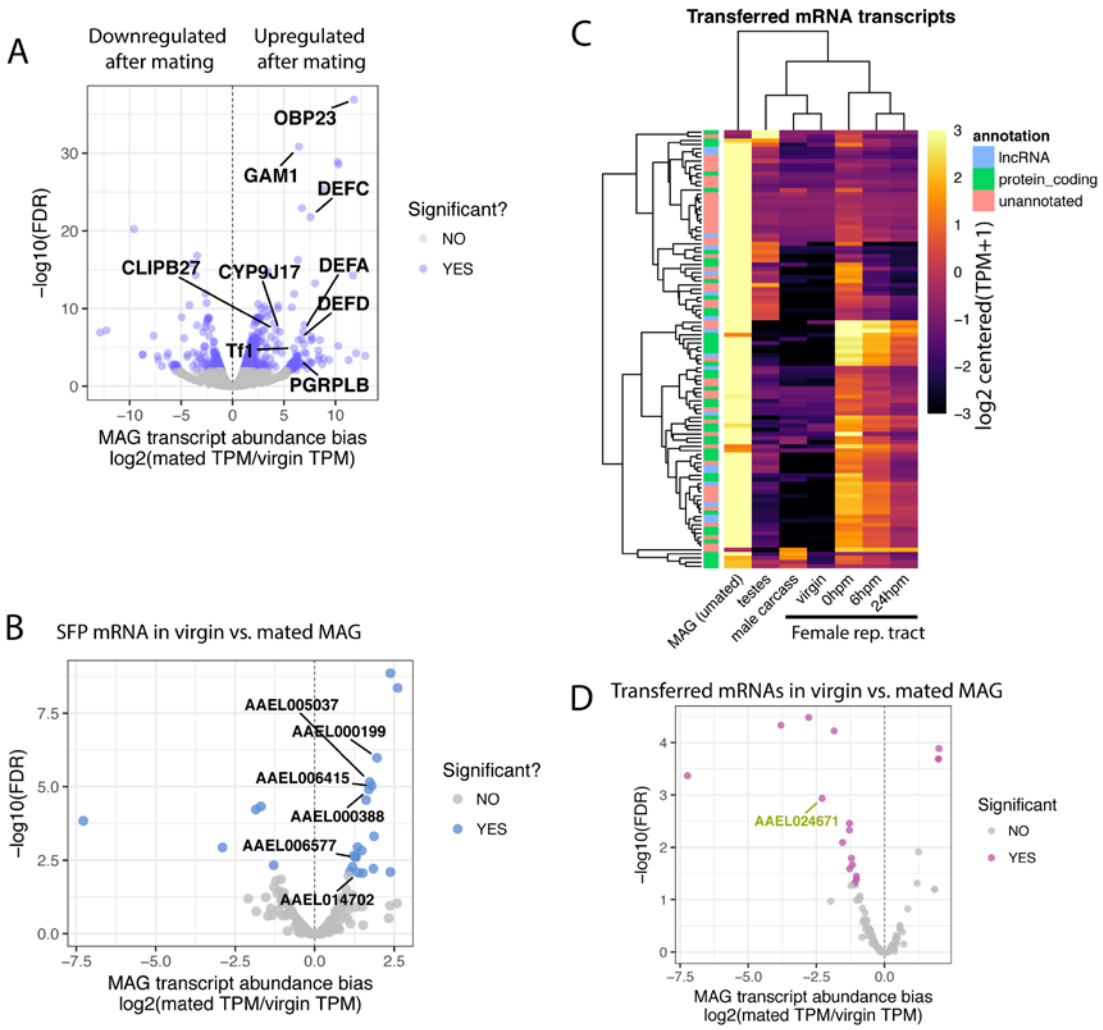


Figure 5.4. Differential expression between virgin and mated MAGs and abundance of transferred mRNAs. (A) Volcano plot of the 446 differentially abundant RNAs between virgin and mated MAGs. Immune-related genes upregulated after mating are highlighted. (B) Volcano plot displaying differential expression of SFP-encoding RNAs between virgin and mated MAGs. tRNA ligase transcripts that are upregulated after mating are highlighted. (C) Heatmap of the 106 transferred RNAs in the MAG and testes samples, female reproductive tract samples (53), and a gonadectomized male carcass sample (52). The annotation classification of each transcript is indicated on the left. (D) Volcano plot of the 106 putatively transferred mRNAs and their differential expression status between virgin and mated MAGs. A single lncRNA that shows reduced abundance after mating is indicated.

were upregulated after mating and only five that decreased in abundance (binomial test; $p < 0.001$; Figure 5.4B). Interestingly, upregulated SFPs included six different cytoplasmic tRNA ligases and a catalytic subunit of a signal peptidase responsible for post-translation removal of secretion signal peptides, consistent with the importance of protein production and secretion in the mated MAG (see Discussion).

Paternal mRNA transfer during mating

Previously, Alfonso-Parra et al. (53) demonstrated that males transfer mRNA to females in the ejaculate. Using the newly annotated genome (42), we re-analyzed data from those experiments and identified 106 transcripts, including 41 RNAs that encoded proteins and 17 long non-coding RNAs, that are putatively transferred to females. Identification was based on >2-fold increase in transcript abundance in females immediately after mating, followed by a subsequent decrease in transcript abundance. The remainder of the identified transcripts (48 of 106) are currently unannotated. Using the transcriptomic data in the current study, we determined that the vast majority of these transcripts have MAG-biased expression, with only two transcripts exhibiting testis-biased expression (Figure 5.4C). The MAG therefore appears to be a primary source of RNA transferred to females in the ejaculate. In total, 27 proteins encoded by transferred mRNA transcripts were identified in our proteomes, including 22 in the high-confidence SFP proteome, three in the sperm/SFP overlap proteome, and five in the sperm proteome. Interestingly, the putatively transferred transcripts whose products were present in our seminal fluid proteome encode highly abundant proteins that were on average six times more

plentiful than the remainder of the seminal fluid proteins. Lastly, transferred transcripts exhibited a general trend towards down-regulation in the MAGs of mated males compared to MAGs of virgins, including 13 transferred transcripts that were significantly down-regulated after mating between virgin and mated MAGs (Figure 5.4C); this pattern may be due to their transfer without replenishment in the MAG by the time of dissection.

SFPs are enriched on chromosome 1

Sex chromosomes present exclusively in males, such as the mammalian and insect Y chromosome, are highly enriched for genes with male-biased function, including many critical to spermatogenesis and sperm function (32, 71, 72). Although *Ae. aegypti* lack heteromorphic sex chromosomes, chromosome 1 harbors a region of robust linkage disequilibrium surrounding the recently characterized male sex determination locus, *Nix* (59, 73). To assess the enrichment of male-biased genes on this chromosome we examined the physical distribution of SFPs, sperm proteins, and MAG/testes-biased genes by chromosome. This revealed a ~1.5-fold enrichment of SFPs on chromosome 1 (62 observed, 39 expected; Chi-square = 12.4, $p < 0.001$) and ~1.2-fold enrichment of sperm proteins on chromosome 2 (377 observed, 316 expected; Chi-square = 11.6, $p < 0.001$), whereas MAG- and testis-biased transcripts showed no such enrichment on any chromosome (all Chi-square < 3.84, $p > 0.05$; Figure 5.5). Despite the strong enrichment of SFPs on chromosome 1, we did not observe an overrepresentation of SFPs in the linked region in the vicinity of the male etermining locus, *Nix* (15 observed, 16 expected; Chi-square = 0.08, $p = 0.8$). Non-

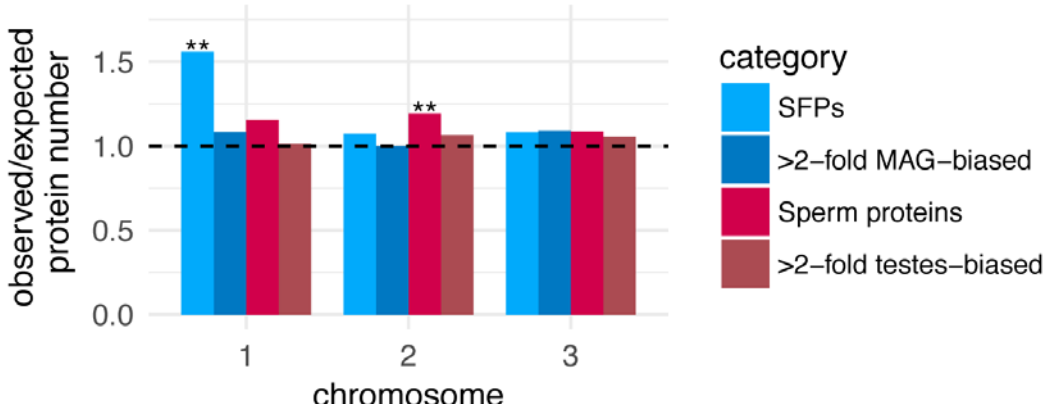


Figure 5.5. Chromosomal distribution of genes encoding SFPs, sperm proteins, or transcripts with MAG biased expression or testis biased expression. The *y*-axis represents the ratio of observed/expected number of genes for each of the chromosomes in *Ae. aegypti*, and the dashed line represents the expectation under no enrichment/depletion. Asterisks indicate significant enrichment (Chi-square; $^{**}p < 0.01$).

uniform physical distribution of sperm proteins across autosomes has also been observed in *Drosophila* (68). Although the functional significance of sperm proteins' clustering on chromosome 2 remains to be determined, it is possible that this pattern facilitates co-expression during spermiogenesis, which is characterized by progressive genome silencing during the histone-to-protamine repackaging transition.

Discussion

A rapidly expanding body of evidence supports the critical roles of seminal fluid proteins (SFPs) in a wide array of reproductive phenotypes (reviewed in 29). Although this has been most extensively investigated in *Drosophila*, seminal peptides and proteins are also associated with post-mating behavioral and physiological responses in mosquitoes such as *Ae. aegypti* (15, 16, 18). The primary goals of this study were to comprehensively catalog male proteins transferred to *Ae. aegypti*

females during insemination and establish a reliable methodology for delineating between sperm proteins and SFPs. To accomplish this, we (**1**) conducted an in-depth proteomic characterization of sperm, (**2**) utilized a whole-female labeling approach to identify unlabeled male proteins transferred by the male during insemination and (**3**) characterized the transcriptomes of the testis and male accessory gland (MAG). Importantly, we note that the whole-female labeling approach has been employed previously in *Ae. aegypti* but the assignment of proteins as SFPs was limited by the lack of information regarding proteins found in sperm. Thus, distinctions between sperm proteins and SFPs were previously difficult to achieve. In the present study, we have re-assigned 39 proteins identified by Sirot et al: 23 previously identified SFPs and 4 sperm proteins as putative components of both sperm and seminal fluid, 10 previously identified SFPs as putative sperm proteins, and 2 previously identified sperm proteins reassigned as high-confidence SFPs. We acknowledge that our MS/MS-based approach still includes some inherent uncertainty, and that this should be kept in mind when interpreting our classifications. It is also noteworthy that advances in MS/MS sensitivity and accuracy have resulted in far greater power of detection in our study, and our analysis has also benefited tremendously from the recent resequencing and reannotation of the *Ae. aegypti* genome (42). Our proteomic characterization resulted in a nearly four-fold expansion of the current *Ae. aegypti* seminal fluid proteome, and an eight-fold expansion of identified proteins in sperm. Together, this *Ae. aegypti* "ejaculatome" provides a foundation for future molecular studies of mosquito reproduction and associated applications to control mosquito populations (see below).

Proteome characteristics independently validate identification

Our work differs from previous SFP characterization studies (27, 28, 36) in that our classification was supported by a detailed knowledge of sperm proteome composition. Nonetheless, several independent validation approaches were helpful in assessing the quality of our proteomic characterization. For example, we quantified the proportion of proteins with predicted secretion signals and analyzed transcriptome profiles in testes and MAGs. As would be predicted, SFPs identified in this study possessed a significantly higher proportion of predicted secretion signals than sperm proteins. While only 33% possessed predicted secretion signals in our high-confidence SFPs, this proportion is consistent with what has been reported in seminal fluid of the grasshopper *Melanoplus sanguinipes* (74). The large proportion of proteins that lacked this signal may be due to the fact that much seminal fluid secretion in *Ae. aegypti* has been reported to occur through both apocrine and holocrine mechanisms (64, 65). The genes encoding high-confidence SFPs were also, on average, highly specific or biased towards expression in the MAG. RNAs encoding over 99% of the identified proteins were also represented in their target tissues' transcriptomes, adding further validation to their identification as seminal fluid and sperm proteins. Those 11 proteins with no expression in their target tissue may represent proteins that were produced outside of the testes or accessory glands (e.g., in adjoining tissues such as the vas deferentia or trafficked into these organs) or transcripts that were not expressed at the time of tissue dissection.

Analysis of the functional composition of our proteomes revealed that they were closely aligned with the results of previous sperm (32-34, 68) and SFP studies

in insects (30, 36). For example, our expanded sperm proteome was highly enriched for proteins related to flagellar structure, including microtubules, dynein complexes, and ciliar components, and proteins likely associated with the mitochondrial derivatives, which are a predominant structure in mosquito sperm (75, 76) and that of other insects. Consistent with what was described by Sirot *et al.* (27), as well as in other insects (reviewed in 29, 30, 36) and humans (77), proteases were highly enriched amongst our high-confidence SFPs, supporting the likely accuracy of our expanded characterization (reviewed in 69). The observed enrichment of vesicle-mediated transport proteins is also consistent with the fact that *Ae. aegypti* seminal fluid is in part produced by apocrine secretion (64). Additionally, exosomes and other vesicles are believed to play a role in a variety of post-insemination cellular interactions. For example, vesicles transferred in *Drosophila* seminal fluid have been reported to fuse with sperm and interact with the female reproductive tract (78), exosomes of the mouse epididymis have recently been implicated in the control of sperm RNA stores (79), and the abundance of exosome markers in avian SFPs has led to speculation about vesicle-mediated mechanisms in post-testicular sperm maturation (80). Therefore, the accuracy of our expanded proteomic characterization of sperm and SFP proteomes is corroborated by several independent lines of evidence.

It is important to note that, despite the application of stringent proteomic thresholds, some proteins could not be definitively assigned as either sperm protein or SFP. Previous studies in *Drosophila* and Lepidoptera have consistently identified known SFPs (such as *Drosophila* Acp36DE) at appreciable abundance levels in

sperm that have yet to be combined with MAG secretions (32, 67, 68). Our identification of a relatively large protein set that is highly MAG-biased in expression but also present in sperm further suggests that the incorporation of "SFPs" during testicular sperm maturation occurs and is worthy of additional functional investigation. Although *Drosophila* expression profiles in the testis and accessory gland are quite distinct, many SFPs exhibit low levels of co-expression in the testis (Dorus, unpublished data). Our transcriptomic analyses here further support such patterns of co-expression. As such, dichotomous distinctions between sperm proteins and SFPs may be an oversimplification of a more nuanced relationship between these reproductive systems. We acknowledge this ambiguity in our classification of MAG-biased proteins that were also identified in our sperm proteome. We also note that our sperm purification method could have allowed the inclusion of seminal fluid proteins (that is, non-sperm ejaculate proteins) that are produced in the seminal vesicle, vas deferentia, or testes. Similarly, the possibility exists that some male accessory gland proteins may migrate into the seminal vesicle. While the contribution of such proteins to the sperm proteome, if any, is likely small, we cannot rule out this uncertainty.

We also note that despite our expanded proteomic coverage, several proteins that we anticipated to be identified were absent. The most notable case was Head Peptide-1, a seminal fluid peptide which has been shown to be transferred in the ejaculate (81) and has been reported to induce short term monogamy in the female after mating (15). Head Peptide-1, like many SFPs, undergoes extensive post-translational modification and may therefore be challenging to identify bioinformatically without *a priori* knowledge of the biochemical composition of the

proteolytic products (such as in the case of the well-studied Drosophila Sex Peptide; 82). Another example was adipokinetic hormone (AAEL011996), which did not meet our two unique peptide inclusion threshold, although we did identify five copies of one peptide from its precursor protein that was also identified in *Ae. albopictus* seminal fluid (28). This protein has been postulated to contribute to sperm protection from oxidative stress (83) and the regulation of feeding behavior (84) in other insects. We suggest that the complexity of proteolytic pathways, governed both by male and female interacting proteins, is a major barrier in the use of shotgun proteomics to study SFP identity and function in the female reproductive tract. Conducting similar analyses with an alternative digestive enzyme or *de novo* peptide sequencing may allow the detection of additional proteins whose tryptic products could not be identified using standard database searches. Searching for proteins in the supernatant of our sperm samples may also identify soluble proteins that associate with the surface of sperm, as has been shown with sex peptide in *D. melanogaster* (85). Finally, future investigations would also likely benefit from the inclusion of a targeted proteomic approach (reviewed in 86). Such approaches require an *a priori* list of candidate peptides; in *Ae. aegypti*, the neuropeptides and protein hormones catalogued by Predel *et al.* (87) represent a useful pool of potentially important molecules.

Evolution of male reproductive proteomes

Male reproductive proteins, including SFPs, are consistently among the fastest evolving classes of protein (reviewed in 88). Although initially a goal of our study,

conducting a robust analysis of the molecular evolution of proteins identified in this study was limited by the availability of genomic resources appropriate for both inter- and intraspecific tests of positive selection. Obtaining high quality genomic data for different populations of *Ae. aegypti* has proven difficult, given the genome's repetitive nature (42, 89). Furthermore, this mosquito's ability to move globally as diapausing eggs has allowed for frequent mixing and a complex population structure (90, 91). The development of appropriate population level genetic data for the analysis of recent selective sweeps should be a priority in *Ae. aegypti*, as it has been in *Anopheles gambiae* (92, 93). Furthermore, given the extent of molecular divergence between *Ae. aegypti* and *Ae. albopictus* (28), the development of genomic resources for a more closely related outgroup to *Ae. aegypti* will assist in understanding evolutionary patterns at the gene level. Despite these limitations, our analysis of orthology did reveal that the suite of proteins contributing to seminal fluid, but not sperm, has diverged substantially from other Dipterans. Although sperm proteins and SFPs possess levels of orthology to the *Drosophila* genome that are comparable to the genome as a whole, only 59% and 4% of orthology was observed when comparing the *Ae. aegypti* sperm and SFP proteomes (respectively) with those of *Drosophila* (32, 36). While some of this disparity may be attributed to differences in overall proteome size and coverage, such a stark contrast is nonetheless compelling evidence of tissue-specific evolutionary patterns. Orthology between *Ae. aegypti* SFPs and *Ae. albopictus* SFPs (28), while more extensive (43%), was still lower than orthology between the sperm proteomes of *Ae. aegypti* and *D. melanogaster*—two distantly related Dipterans. These general patterns of orthology among *Ae. aegypti*,

Ae. albopictus, and *D. melanogaster* are consistent with those described by Boes et al. (28), although the absolute level of orthology between studies varies considerably due to methodological differences. The patterns we observe suggest a process of "turn-over" in seminal fluid proteomes, whereby overall protein composition diverges rapidly even when there is evidence for conservation with regard to overarching molecular functions represented in seminal fluid. For example, *a priori* expectations about Gene Ontology enrichment were met for both *Ae. aegypti* sperm (e.g., cilium and mitochondrial proteins) and SFPs (extracellular localization and hydrolase activity), despite overall SFP divergence. SFPs are a pronounced target of selection and have been discussed as a driver of sexual conflict (reviewed in 94), and thus they are expected to rapidly diverge. By contrast, we note that strong conservation of sperm proteins exists across distant taxa, with different insect orders displaying 25% orthology between sperm proteomes (34), and even *D. melanogaster* and mammals with 20% sperm proteome orthology (32). The overall lack of conservation in seminal fluid proteomes makes comparing the roles of specific SFPs across species difficult, but conserved molecular functions amongst SFPs will nevertheless allow the wealth of knowledge in *Drosophila* to be leveraged towards an understanding of SFP function in non-model insects.

Unlike other mosquitoes with heteromorphic sex chromosomes, Culicine mosquitoes (e.g., *Aedes and Culex*) harbor male-determining loci on undifferentiated, homomorphic sex chromosomes (95). Theory predicts the evolution of heteromorphic sex chromosomes following the acquisition of a sex determining locus, suppression of recombination, and expansion of the non-recombining region. It remains unclear

why homomorphic sex chromosomes appear to be retained in some taxa (96, 97). One proposed mechanism to mediate the selective effect of sexually antagonistic alleles on the promotion of recombination suppression is the establishment of efficient sex-biased expression (98). Although previously lacking, the significant enrichment of SFPs on chromosome 1 is the first evidence in support of this hypothesis in *Ae. aegypti.* This trend was restricted to SFPs and was not observed for genes solely over-expressed in the MAG or testis. It is intriguing to speculate that this distinction between SFPs and other male reproductive genes might be due to the prevalence (and selective strength) of sexually antagonistic alleles specifically amongst SFPs, which may favor their localization on chromosome 1. This is consistent with their putative role as drivers of sexual conflict (reviewed in 94), including the mediation of female post-mating responses such as sexual receptivity and longevity (14, 18).

Functional relevance of abundant sperm proteins and SFPs

Our sperm and SFP proteomes exhibited skewed abundance distribution with the top ten most abundant proteins comprising 25% and 17% of protein composition in sperm and SFPs, respectively. Interestingly, the most abundant sperm protein, cytosol aminopeptidase (AAEL006975), accounted for more than 7.4% of all protein and two other cytosol aminopeptidases were in the top ten most abundant proteins (AAEL000108, AAEL023987). These three proteins are orthologs of the eight sperm-leucyl aminopeptidases (S-LAPs) in *Drosophila* with similar expression patterns, including ~1000-fold higher expression in testes than in MAG, ~50 times

more transcript in whole male carcasses than gonadectomized carcasses (99), and upregulation during later stages of spermatogenesis (52). S-LAP orthologs constitute a significant proportion of the protein composition of *Drosophila* (100) and Lepidoptera (34, 67). Little is known about the specific function of S-LAPs, although it has been postulated that may serve a structural function given the inferred loss of enzymatic capacity of several S-LAPs during *Drosophila* evolution (100). Additionally, a Y-linked S-LAP in *D. pseudoobscura* has been implicated in a cryptic meiotic drive system, where suppression of this locus results in aberrant spermatogenesis and a higher proportion of X-bearing sperm (101). It will be of great interest to establish the specific function of these proteins in *Ae. aegypti* sperm, given their high abundance and expression patterns during spermiogenesis. Furthermore, the proteins and transcripts involved in spermatogenesis described in this study may assist in the identification of other genes involved in meiotic drive systems (reviewed in 102), which have been proposed as potential genetic means to reduce wild populations through the induction of sex ratio biases (103).

Although no SFP was as abundant as cytosol aminopeptidase in sperm, the top ten most abundant SFPs ranged from 1.2 – 2.6% of the protein in our ejaculate sample. L-asparaginase (AAEL002796) was the most abundant SFP (61% more abundant than the next protein) and the tenth most abundant mRNA transcript in the MAG out of over 11,000 transcripts. While the relevance of the abundance of this enzyme is currently unclear, it may relate to several other notable observations. First, transcript AAEL020035, whose protein product is comprised of ~60% asparagine residues, is the single most abundant MAG transcript and was also, by far, the most

abundant putatively transferred transcript. (We did not identify AAEL020035 in our SFP proteome but note that it results in few identifiable peptides because of its extreme amino acid composition). Second, asparagine tRNA-ligase (AAEL006577) was two times more abundant in seminal fluid than any other tRNA-ligase. Third, asparagine tRNA-ligase was upregulated in the MAG after mating and was the most abundant tRNA-ligase transcript. Together, these suggest that MAGs are well-equipped to produce ample protein with a strong asparagine amino acid bias. Finally, two other enzymes, aspartate transaminase (AAEL002399) and citrate synthase (AAEL004297), are abundantly present in seminal fluid and could convert aspartate produced by asparaginase to oxaloacetate and citric acid, respectively. While it is premature to draw any firm conclusions based on these observations alone, it is intriguing to speculate that the SFP proteome has the capacity to conduct gluconeogenesis (of asparagine and potentially other amino acids) and that this may feed into to the citric acid cycle. This hypothesis is supported by the results of our KEGG analysis, in which carbon metabolism, gluconeogenesis, and alanine, aspartate, and glutamate metabolism were enriched in the seminal fluid proteome. The citric acid cycle is believed to be functional in mammalian sperm (reviewed in 104), and our KEGG analysis reveals an enrichment of citric acid cycle enzymes in sperm but not seminal fluid. Whether metabolite precursors to the citric acid cycle are transported from seminal fluid to sperm remains to be determined.

The most abundant seminal fluid proteins also exhibited a strong enrichment for function in protein cleavage. Proteins with protease, dipeptidase, and aminopeptidase activity represent 12 of the top 28 most abundant proteins present in

the seminal fluid proteome. Proteolytic functions have been described previously in the seminal fluid of *Ae. aegypti* (27), *Ae. albopictus* (28), *Cx. quinquefasciatus* (105), and several non-mosquito taxa (21, 22, reviewed in 69, 106), and are a common function of many insects' seminal fluid. Based on studies in other insects, functions of these enzymes may include the activation of sperm motility or the cleavage of propeptides into their active forms (107). Our seminal fluid proteome also contains abundant enzymes that catabolize smaller substrates, such as amino acids and carbohydrates. Taken together, the enzymatic cocktail in seminal fluid may be well equipped to break down many of the molecules they contain. Seminal fluid proteins were also enriched for proteins involved in maintaining proton and redox homeostasis. We identified several proteins contributing to V-type proton ATPases, which use ATP to regulate pH via proton transport. Maintaining an optimal pH in seminal fluid may allow for efficient sperm motility (reviewed in 108). Regulating pH may also create an ideal environment in which enzymatic reactions occur, either in organelles such as phagosomes and lysosomes (whose constituents were enriched in our KEGG analyses), in sperm, or in the extracellular environment. Seminal fluid also contained several proteins that function to neutralize free radicals, such as catalase (AAEL013407), peroxidase (AAEL013171), and several dehydrogenases. Regulating the physiochemical environment in seminal fluid is likely critical for the function and protection of sperm prior to their storage in the female's long term storage organs (spermathecae).

Ejaculate RNAs Transferred to Females

There has been much conjecture about the importance of spermatozoal RNA to fertility (109), and recent work has confirmed that the regulation of sperm ncRNA stores in the mammalian epididymis is necessary for proper embryogenesis (110). Little is known about the function of spermatozoal RNAs in insects, although they have been demonstrated to have substantial functional coherence, including an overwhelming enrichment of loci involved in translation (111). New data in this study allowed us to probe for patterns in previously described transcripts that are putatively transferred to females during mating. A total of 106 transcripts were identified, including both coding and non-coding transcripts, and a majority of these exhibit high levels of expression in the MAG. Based on our SFP proteome, most of the protein coding transcripts are also translated at high levels. Their high expression in the MAG suggests that they may simply hitchhike into seminal fluid with other secreted molecules. Alternatively, as has been demonstrated in *Drosophila*, they could be transferred in intact MAG cells (66), or via vesicles derived from the MAG (78). Interestingly these vesicles, which may carry RNA cargo including miRNAs, fuse with sperm and have the capacity to interact with the female reproductive tract. Some male-derived transcripts are detectable in the female for up to 24 h post-mating (50), and it has been postulated that they could be used by females in some capacity (112). In *Ae. aegypti*, both vesicles and RNAs are transferred in the ejaculate to the female, but their fate and function have not been investigated. Whether they impact the female or her future offspring is an intriguing, and potentially important, line of future investigation.

Mosquito control and future directions

Understanding the molecular architecture of Ae. aegypti reproduction holds great potential for vector control strategies. Mosquito reproduction is an ideal control target to reduce vector populations and the burden of disease transmission. The most direct application of this study will be the identification of modulators of female reproductive behavior. Mosquito SFPs induce behavioral responses that prevent female remating (14, 16, 17), including short term mating refractory behavior (15). To date, the molecule(s) responsible for long term refractoriness has yet to be identified. Given the strength and duration of responses to low SFP "doses" (14), identification of the responsible proteins will provide powerful tools for manipulating female reproduction in a species-specific manner. In addition, such knowledge may provide a molecular metric by which the quality of males in modified mosquito release strategies (such as those employing sterile or Wolbachia-infected males; reviewed in 113) may be monitored and optimized. Functional analysis of specific sperm proteins and SFPs may yield insights into processes such as sperm motility and activation (21-23), sperm storage (114), and sperm-egg recognition (115). Very few studies have explored these processes in *Ae. aegypti* (reviewed in 116). A mechanistic understanding of complex post-copulatory male-by-female interactions is critical to genetically modified mosquito release strategies that manipulate reproduction. Our detailed characterization of the male contributions to these interactions should serve as the foundation for the design and improvement of vector control strategies that limit the transmission of arboviruses that cause serious human illness and mortality.

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CHAPTER 6

RESTING, MATING, AND FEEDING BEHAVIOR OF *AEDES AEGYPTI IN* MEDELLÍN, COLOMBIA[*]

Introduction

Aedes aegypti mosquitoes are a serious threat to human health and life in the tropics and sub-tropics. Being the primary vector of the dengue virus, these mosquitoes have been a consistent and growing threat for several decades[1]. More recently, they have driven the resurgence of yellow fever[2] and caused frightening epidemics of formerly obscure ailments, such as chikungunya[3] and Zika[4, 5]. Despite occasional success in campaigns to eradicate[1], *Ae. aegypti'*s geographic mobility[6] and intimate association with humans[7] have nonetheless allowed its continued reign as the most dangerous arbovirus vector on Earth[8]. It is clear that new solutions are necessary for controlling these mosquitoes and the viruses they transmit.

Promising strategies to reduce *Aedes*-borne arboviral diseases are under development or being field-tested. Examples of such mosquito control methods include: population replacement scenarios using *Wolbachia*, with the goal of driving a dengue-resistant phenotype into a population[9]; variations on sterile insect technique using *Wolbachia* or genetic modification to suppress populations[10]; and biological

[*] This work represents a collaboration with Laura C. Harrington, Catalina Alfonso-Parra, and Frank W. Avila. ECD conceived of experiments, directed and coordinated collections, conducted collections (with the help of field assistants Paulina Gutiérrez Arbeláez, Sindy Carolina Bedoya Patiño, and Edison Estiven Trujillo Gil), processed and analyzed all specimens, analyzed all data, and wrote this chapter. LCH conceived of experiments, provided critical guidance to their implementation, and critically revised manuscript. CAP and FWA provided essential material and logistical support and helped design experiments.

control using entomopathogenic fungi or yeast-based RNAi delivery systems[11]. The success of all of these technologies relies not only on effective control mechanisms, but also an intimate knowledge of how such mechanisms will function and play out in a field setting.

The knowledge base of *Ae. aegypti* biology in diverse field settings lags far behind that of *Ae. aegypti* in laboratory-adapted colonies maintained with unnatural diets and climates. We do not dispute that laboratory-based, experimental studies are important drivers of our understanding of mosquito biology. However, field-based studies are critical to translating laboratory-tested entomological findings into epidemiological successes in the field. Investigations in the field should span diverse epidemiological settings to better understand this ecologically plastic mosquito.

In this study, we aimed to understand female *Ae. aegypti* feeding, resting, and mating behavior by intensively sampling the adult population of indoor mosquitoes in Medellín, Colombia. We recorded the resting location and physiological state (amount of blood feeding, egg development, and mated status) of *Ae. aegypti* females. We also recorded demographic data from each dwelling in which mosquitoes were collected in order to understand predictors of mosquito presence and absence. Comparing our data to previous reports of *Ae. aegypti* resting behavior, we find similarities and key differences that may represent strain- or geography-specific bionomics. We also provide evidence for facets of mosquito reproductive biology that formerly had weak or no support from studies in the field. We emphasize that understanding the nuances of mosquito biology in a given locale will be critical to the success of mosquito control.

Methods

Site selection and description

All collections were conducted in Medellín, Colombia, in the districts ("comunas") of Popular and Santa Cruz. These adjacent communities in Medellín were chosen based on their high endemicity for dengue as well as their high density of *Ae. aegypti* mosquitoes. They are densely populated, urban communities in the lowest of six cadastral levels (i.e., socioeconomic levels defined by the city for taxation and public works purposes). Homes in these communities typically consist of one level of a one, two, or (more rarely) three story building. The majority of the open space in these communities is composed of impervious surface, with few homes having gardens or yards. Homes are adjacent to each other and are often built up a steep mountainside. Therefore, streets and homes often follow the contours of the mountain or occasional creeks.

Because the goal of this study was primarily to understand individual mosquito behavior and physiology (and not, for example, to understand the spatial distribution and density of vectors in Medellín), collection sites were not chosen randomly. Rather, investigators chose locales with the goal of capturing as many mosquitoes as possible. Collectors focused their efforts on locations adjacent to creeks and where residents had informed them of a home that often has many mosquitoes. After an initial location was chosen each day, investigators proceeded down a street or through enclaves of homes, seeking permission to collect in each home. In total, 835 different homes were searched for adult mosquitoes. All collections were conducted between the hours of 9:00 am and 4:00 pm from April 2nd – July 18th, 2018. The Cornell University IRB

determined that mosquito and data collection (Protocol ID: 1801007702) did not meet the definition of "human participant research," and thus no IRB approval was necessary.

Mosquito collection

All collections were conducted with permission from the Medellín Secretary of Health. Preliminary collection efforts in outdoor spaces (such as public parks or backyard gardens) yielded very few mosquitoes. Therefore, we focused our efforts entirely on indoor collections to target the endophilic *Ae. aegypti*. At each home, collectors sought permission from an adult resident to collect in the house by explaining the general purpose and method of the investigation. Collectors systematically worked through each room of each home (unless the homeowner requested they not enter certain rooms) collecting mosquitoes with handheld aspirators[12]. They aspirated all surfaces (including ceilings, walls, any household objects, under beds, etc.) that the collector could safely reach, using a 1.2 m extension pole when necessary. Small chairs were moved to allow aspiration, but other furniture was left in place. The outflow of the aspirator was blown into confined spaces that could not be reached to encourage resting mosquitoes to fly. Accessible surfaces were only excluded if investigators could plainly see that there were no mosquitoes present (for example, clean, white, painted walls). The start time and overall collection effort (measured in person minutes) were recorded for each home. After aspirating, mosquitoes were transferred into microcentrifuge tubes and placed on wet ice. The

number of residents dwelling in the home, the number and type of pets present, and on which story the home was located were recorded in each location.

After one month of collection (245 houses), the decision was made to record the room in which mosquitoes were captured and their exact resting location in those rooms. These data were only recorded if the collector was certain of their accuracy. Therefore, mosquitoes were excluded from resting data if the collector did not notice their capture until after the aspirator was turned off. Similarly, collections that included mixed males and females or multiple species were excluded, unless all mosquitoes were collected in the same room or the collector was certain of a mosquito's identity as it was being collected. If a mosquito was captured in flight, its room was recorded but no exact resting location was recorded. Therefore, of 566 female *Ae. aegypti* collected in this period, room data were recorded for 365 and exact resting location for 176 mosquitoes.

For the final month of collection, collectors also recorded whether mosquitoes were collected above or below 1.5 m of height (as in [13]). Collectors mentally marked this precise height on their body (e.g., at outstretched horizontal arm level, or nose level, depending on the collector) and judged whether mosquitoes were collected above or below this height from the floor. Similar to above, this variable was only recorded when collectors were certain of a mosquito's resting location. These data were recorded for 103 of 227 female *Ae. aegypti* collected. During this final month, the type and number of rooms that each house contained was also recorded (n = 233 homes).

Mosquito processing

In the laboratory, mosquitoes were examined for the presence of blood. Meals that were still red were noted. The proportion of their abdominal cavity that was filled with blood was also recorded, as a measure of the degree of digestion of the blood meal[14]. If any blood was present, the maximum distance of integument visible between the dorsal tergites and the ventral sternites was measured as a proxy for the degree of engorgement of each female. This inter-sclerite distance (ISD) was measured by superimposing the midleg's femur at its widest point against the exposed integument, and counting the number of femur widths (FW, to the nearest half unit) of integument exposed between sternites and tergites. This method was used as a means of quickly scaling engorgement to body size. A pilot experiment demonstrated that hand-measured ISD:FW ratios are 98% accurate, and the maximal engorgement of females varies little with body size (see below).

After scoring blood meals, females' spermathecae and ovaries were dissected. Spermathecae were checked for sperm to ascertain whether females had mated; females typically store more than 1000 sperm, and thus any female that had mated is highly unlikely to have used all of her stored sperm[15]. Ovaries were scored for either parity (i.e., whether females had laid eggs or not)[16] or Christophers' stage of egg development[17]. The number of eggs produced was counted if females had eggs at stage V (fully developed) or IVb (fully enlarged and at final shape, but without tubercles). Finally, if the female had any blood it was placed into a microcentrifuge tube and kept at -80°C for future analysis.

Verification of ISD:FW ratio as a measure of engorgement

Two groups of females were reared: normal-sized females (reared according to the methods of [18]), and small females (reared in the same manner but increasing the density of larvae 3.5-fold). From these, three groups were blood fed and assayed for engorgement via their ISD:FW ratio: normal-sized females at 6 dpe, normal sized females at 20 dpe, and small sized females at 6 dpe. For each group, females were fed human blood on the arms of ECD for 10 min. Females were placed on wet ice 15 min after cessation of feeding to prevent diuresis while they were being processed and measured. A wing was removed from each female and measured as in [19]. Next, engorgement was measured with the mid-femur as described above. Finally, the

Table 6.1. Femur widths and inter-sclerite distance to femur width (ISD:FW) ratios. All treatments have minimum $n = 27$. Values given are mean ± standard deviation.

Treatment	**Femur width (µm)**	**Engorgement (ISD:FW)**		**Accuracy**
		Hand-measured	*Digitally measured*	
Small 6 dpe	116.6 ± 7.5	6.62 ± 0.49	6.72 ± 0.61	0.95 ± 0.08
Normal-sized 6 dpe	161.2 ± 12.5	6.52 ± 0.68	6.19 ± 0.73	1.03 ± 0.09
Normal-sized 20 dpe	161.0 ± 6.8	5.65 ± 0.84	5.58 ± 0.77	0.99 ± 0.07
			Overall	0.98 ± 0.09

female's abdomen was photographed while she was laying on her side, and the female's midleg was also photographed. Finally, the ISD and FW were measured digitally. Overall, hand-measured and digitally measured ISD:FW ratios were not significantly different from each other (Table 6.1). Using digitally measured ISD:FW as an engorgement metric, old females engorged to a smaller degree than young females (Welch's *t*-Test; $t = 2.79$; $p = 0.007$). Small females engorged 8.7% more than large females of a similar age (Welch's *t*-Test; $t = 3.17$; $p = 0.002$). Femur width did not differ between young and old normal-sized females (Welch's *t*-Test; $t = 0.07$; $p = 0.97$), whereas small females had femurs 72% the width of large females (Welch's *t*-Test; $t = 17.37$; $p < 0.001$).

Statistical analysis

Predictors of *Ae. aegypti* female presence: Several homes were sampled more than once, often at the request of owners of homes with many mosquitoes. For house-level summary statistics and analysis of predictors of mosquito-positive homes, only the first of repeated visits to these homes was included. Only 17 homes spanned more than one story, and these were analyzed as located on their lowest story. A generalized linear model (GLM) with a logit link was constructed, with the response variable being the presence of at least one female *Ae. aegypti*. The initial model included number of residents in each home and its story as covariates. Whether each home had cats, dogs, or birds were included as three separate binary factors. The model was run iteratively, removing the least significant term each step until all were significant. Additionally, Pearson Chi-square tests were conducted to test each binary factor

individually and calculate risk and odds ratios for a home having female *Ae. aegypti* present.

Resting behavior: All mosquitoes collected during the study period were included in mosquito level analyses (i.e., resting behavior, mating behavior, and female reproductive physiology), whether they came from a repeatedly sampled home or not. Whether the rooms in which mosquitoes were collected differed from a random distribution across all room types was calculated using a Chi-Square Goodness of Fit test. Resting locations are reported as one of six categories: ceiling, perimeter of the room (i.e., walls, doors, or windows), clothing (including shoes and hats), electronic equipment, furniture, or other objects in the room.

Virgin vs. female feeding behavior and physiology: The proportions of virgin and mated females that had any amount of blood in their midgut were compared using a Pearson Chi-Square test. To compare virgin and mated female blood meal sizes, we first grouped females into similar stages of blood meal digestion, because digestion and diuresis decrease the volume of the abdomen progressively over time after a blood meal. We used the proportion of their abdomens filled with undigested blood as a proxy for blood digestion status (Illustration 6.1). Females were binned into one fourth increments, with the most recently fed females having nearly all of their abdomen full of blood, and females whose blood meal was almost entirely digested having only a small spot of undigested blood visible through the cuticle. Mated and virgin females' ISD:FW were compared within each bin using Mann Whitney U tests. A fifth Mann Whitney U test was used to conduct a similar comparison with females whose blood meal was still red, indicating a recent blood meal. A one-way ANOVA was used to

test for differences in the number of eggs contained by mated females with stage IVb eggs (*n* = 39), mated females with stage V eggs (*n* = 93), and gravid virgins with IVb or V eggs (*n* = 33). Gravid virgins with IVb or V eggs were pooled because they are highly unlikely to have laid any eggs without the ability to fertilize them.

Illustration 6.1. Estimates of the proportion of each female's abdomen with undigested blood were binned into one fourth increments. Illustration demonstrates a female in each bin. Left most mosquito indicates a female whose blood meal was scored as "red."

Results

We aspirated mosquitoes in 835 different homes from April – July 2018. From these, 354 were *Ae. aegypti* positive and yielded 718 female and 388 male *Ae. aegypti*. Of homes in which *Ae. aegypti* were collected, the number of *Ae. aegypti* mosquitoes we captured per positive home ranged from 1 – 58, and was on average 1.85 females and 0.96 males. We also captured 76 *Ae. albopictus* and *Culex* sp. individuals. Because *Ae. aegypti* females were the focus of this study (due to their epidemiological relevance), all subsequent results and discussion focus on these individuals only.

Predictors of female Ae. aegypti *presence in households*

We used a generalized linear model to estimate predictors of the presence of female *Ae. aegypti* in homes (hereafter "mosquito-positive"). Of all tested factors,

Table 6.2. Results of Pearson Chi-square tests of female *Ae. aegypti* presence by different demographic factors associated with homes.

	Negative homes	Positive homes	Risk ratio (95% CI)	Odds ratio (95% CI)	χ^2	*p*
Cat presence	98	65	1.31	1.52	5.44	0.02
Cat absence	468	204	(1.05 - 1.64)	(1.07 - 2.17)		
Dog presence	151	87	1.20	1.31	2.87	0.09
Dog absence	415	182	(0.98 - 1.47)	(0.96 - 1.80)		
Bird presence	57	25	0.94	0.91	0.12	0.73
Bird absence	509	244	(0.67 - 1.33)	(0.56 - 1.50)		
1st floor	316	178	1.22	1.34	2.91	0.09
2nd floor	164	69	(0.97 - 1.53)	(0.96 - 1.87)		
1st floor	316	178	1.57	1.89	4.87	0.04
3rd floor	57	17	(1.02 - 2.42)	(1.07 - 3.35)		

only home level and cat presence were significant predictors of mosquito presence (GLM; likelihood ratio $\chi^2 = 11.514$; $df = 2$; $p = 0.003$). Homes with at least one cat were significantly more likely to be mosquito-positive (GLM; Wald $\chi^2 = 4.211$; $df = 1$; $p = 0.04$). Home level was also significantly associated with female *Ae. aegypti* presence, with lower levels being more likely to be mosquito-positive (GLM; Wald $\chi^2 = 6.654$; $df = 1$; $p = 0.01$). To understand the risk associated with a given factor, we also report results of separate Pearson Chi-Square tests, risk ratios, and odds ratios for these factors (Table 6.2). Homes on the first floor are 22% and 57% more likely to be mosquito-positive than homes on the second and third floors, respectively, although

the former difference is not significant (Pearson Chi-square test; $\chi^2 = 2.91$; $p = 0.09$). While dog presence is also not significant (Pearson Chi-square test; $\chi^2 = 2.87$; $p = 0.09$), homes with dogs were 20% more likely to be mosquito-positive than homes without dogs.

Resting behavior

Of the 365 mosquitoes for which a resting room was recorded, 75% were found in bedrooms, followed by 13% in living rooms, and the remainder in other locations in the house (Figure 6.1). To understand whether this is because of a proclivity for resting in bedrooms or because there were simply more bedrooms present, we also quantified the number of each type of room in 233 homes. While bedrooms were the most common room, the proportion of mosquitoes collected in bedrooms far surpasses the proportion expected if mosquitoes were randomly distributed across rooms (Chi-Square Goodness of Fit test; $\chi^2 = 310.62$; $df = 6$; $p < 0.001$; Figure 6.2).

Of the 176 mosquitoes whose resting location was observed, 60% were found on ceilings and 20% elsewhere along the perimeter of the room (that is, on walls, doors, or windows). The remainder were located on furniture, electronics, clothing, decorations, or other items (Figure 6.2). Finally, for the last month of collection, collectors recorded whether each captured mosquito was resting above or below 1.5 m from the floor. This additional information was added in order to make a direct comparison to the results reported in [13]. Of 103 female *Ae. aegypti* mosquitoes for which these data were recorded, 86% were found to be resting above 1.5 m.

Blood feeding, mating, and oogenesis

Of all of the mosquitoes we collected, 35.3% were virgin and 64.7% were mated (n = 755). In virgins and mated females, similar proportions of females had some amount of blood in their abdomens (82.0% and 78.7%, respectively; Pearson Chi-Square test; $\chi^2 = 1.07$; $p = 0.30$). To assess whether virgins and mated females took different sized blood meals, we measured the amount of exposed integument between the dorsal tergites and the ventral sternites. To normalize for potential size differences of field-collected females, we measured this distance using the width of each female's femur as a unit. Virgins' abdomens were less distended whether we examined females with red blood (indicating a recent blood meal; Mann Whitney U test; $z = 2.574$; $p < 0.001$) or had partially digested blood (Mann Whitney U tests; $z > 2.339$; $p < 0.05$). These differences were observable in females up to the point of digestion at which their blood meal filled less than one fourth of their abdominal cavity (Mann Whitney U test; $z = 0.257$; $p = 0.795$; Figure 6.3; Illustration 6.1). To assess the order of blood feeding and mating, we analyzed the 618 mosquitoes that had either blood fed, mated, or done both. Of all captured mosquitoes, 32% had taken a blood meal but not mated, whereas fewer than 2% had mated but not fed (as evidenced by nulliparity and an empty midgut). The remaining 64% had both mated and taken at least one blood meal.

Finally, we quantified egg production in females. A small proportion (n = 15) of females contained one or two eggs; all such females were mated, are assumed to have simply retained these eggs from a previous gonotrophic cycle, and are therefore excluded them from the following analysis. Of 709 female *Ae. aegypti* collected,

25.5% had either nearly mature (stage IVb) or mature (stage V) eggs. Of these, more than one fifth were virgin. Females had a similar number of eggs whether they were virgin (83.1 ± 24.5 eggs), fully gravid (stage V; 81.9 ± 24.7 eggs), or nearly gravid (stage IVb; 81.6 ± 28.2 eggs) (means ± SD; One-way ANOVA; $F = 0.034$, $df = 2$, $p = 0.97$).

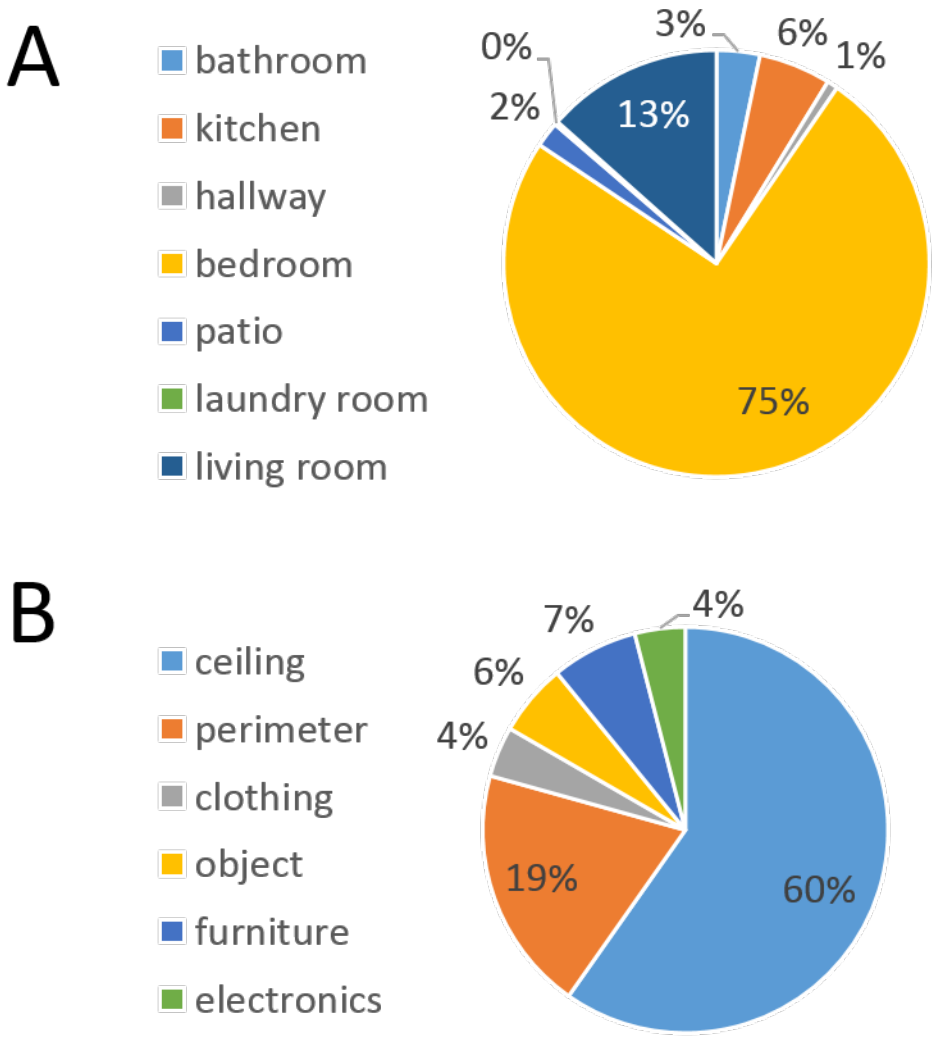

Figure 6.1. Resting location of *Ae. aegypti* females. (A) Percentage of mosquitoes captured in a given type of room (*n* = 365 mosquitoes). (B) Percentage of mosquitoes captured in specific locations (*n* = 174 mosquitoes).

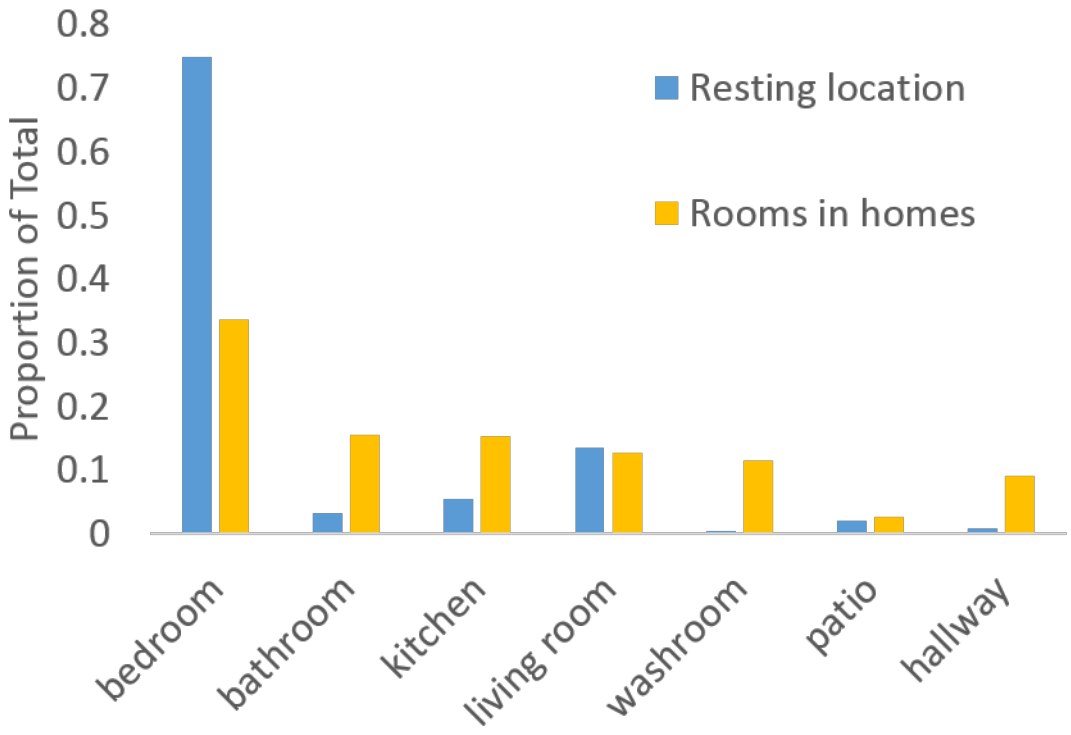

Figure 6.2. Proportion of mosquitoes that were captured in each room type (blue; *n* = 365 mosquitoes) compared to the proportion of each room type that makes up a given home (yellow; *n* = 233 homes). Proportions of home make-up are based on room counts and not area.

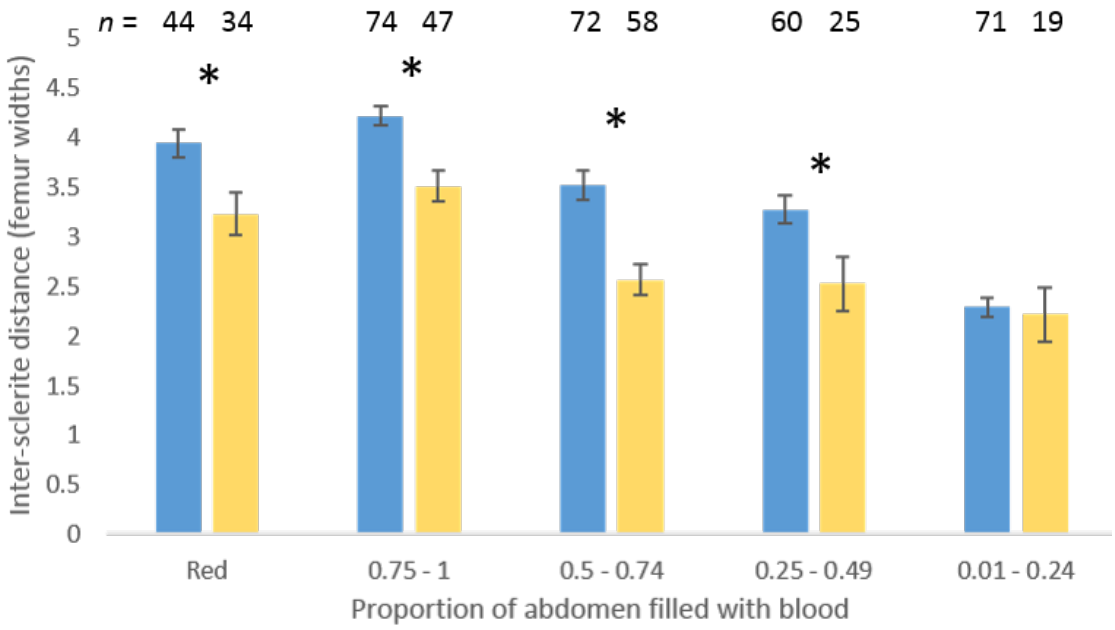

Figure 6.3. Inter-sclerite distance (measured in widths of mid-femur) of mated (blue) and (yellow) females at different stages of blood digestion (as measured by red blood or proportion of abdominal cavity filled by blood). Pairs with asterisk are significantly different (Welch's *t*-test; $p < 0.05$). Sample sizes listed above each group.

Discussion

This was the first investigation of female *Ae. aegypti* feeding, resting, and mating status in Medellín, Colombia. This city has high transmission of dengue[20], yet it differs from some other dengue endemic locales in that it has a consistently temperate climate, with daily low and high temperatures of 17 and 28°C, respectively, year round. Therefore, we conducted this study to better understand the behavior and physiology of *Ae. aegypti* in this unique epidemiological context. We recorded several different types of data, including characteristics of homes that *Ae. aegypti* inhabits, its preferred resting locations within those homes, and the physiological state of females.

Where do we find resting mosquitoes?

Understanding where *Ae. aegypti* rests in the home is an important behavioral trait to consider when designing vector control efforts. Given *Ae. aegypti*'s endophilic and anthropophilic nature[21], indoor residual spraying has been demonstrated to be an effective vector control method for this mosquito[22]. This method can be accomplished more quickly and economically if application can be concentrated on portions of a home on which mosquitoes are most likely to rest.

We found that the presence of cats in homes was significantly associated with female *Ae. aegypti* presence; homes with cats were 31% more likely to be mosquito-positive. To our knowledge this is the first study to report such an association. Dogs increase the likelihood of being positive for female *Ae. aegypti* by 20%, although this association was not significant. Given that *Ae. aegypti* primarily feeds on humans[23], these findings were unexpected. The cause of this positive association with pets is at present difficult to discern. Forthcoming analysis will analyze whether blood fed mosquitoes collected in these homes fed on humans or their pets.

Another factor associated with female *Ae. aegypti* presence was home level. First story homes were 57% more likely to be mosquito-positive than third story homes, and 22% more likely to be mosquito-positive than second story homes (although the latter association was not significant). Several other studies have attempted to quantify the vertical dispersal of *Ae. aegypti* in highrise apartments[24, 25, 26]. The consensus from these studies is that, in general, *Ae. aegypti* does disperse vertically and that there is a weak negative correlation between *Ae. aegypti* presence and height. However, all of these studies used ovitraps to collect females. It may be

that the ample hosts and shelter from wind at ground floors makes adult *Ae. aegypti* more likely to seek out lower levels for feeding and resting in Medellín.

We found that the vast majority of *Ae. aegypti* females rested in bedrooms. This is in agreement with three previous studies in Latin America[13, 27, 28]. While we did not quantify the lighting or ventilation in these rooms, we observed that bedrooms were often dimly lit, on the interior of homes, and had limited ventilation. Such conditions may be ideal for *Ae. aegypti* to rest undisturbed, avoiding the elements as well as the attention of residents while they digest blood meals. An opportunity to bite sleeping hosts may also favor their resting in bedrooms, although *Ae. aegypti* is primarily a day-biting mosquito[29, 30]. Illustration 6.2 exemplifies this preference for bedrooms. In this home, 20 *Ae. aegypti* females were captured in a dark, secluded bedroom. By contrast, only eight were collected in the rest of the home, which included other bedrooms and a large living room, albeit with more lighting and ventilation. Homes in both communities had more bedrooms than any other type of room, but the proportion of bedrooms in homes does not alone explain why so many mosquitoes rest in them.

Within rooms, we found that most mosquitoes rested above 1.5 m, with the most common resting locale being ceilings. As an example, all 20 of the females in the home in Illustration 6.1 were found on the ceiling (n = 17) or above 1.5 m on the wall (n = 3). This is in direct contrast to previous studies in Latin America. Vazquez-Prokopec et al.[12] and Dzul-Manzanilla et al.[13] found that in Acapulco, México, and Iquitos, Perú, 82% of *Ae. aegypti* mosquitoes were found resting below 1.5 m, and Perich et al.[27] found that 60% of *Ae. aegypti* were found resting below 1 m. We

thoroughly collected mosquitoes from all accessible surfaces, including underneath beds. While it is possible that our methods resulted in a biased capture rate between low- and high-resting mosquitoes (for example, because area behind furniture was inaccessible), such differences are unlikely to explain the stark discrepancy between our study and previous work. Rather, we propose that the different climate between Medellín and these other locations may influence resting behavior. Daily temperatures in Medellín typically fluctuate between 17 and 28°C, whereas the climate in these other studies is about 4°C warmer. Future mosquito collections in Medellín would benefit by standardizing the time spent aspirating above and below 1.5 m (as in [13]) to allow more direct comparisons of our results to those of previous studies. Laboratory assays designed to test mosquito temperature preference, such as those used to test the behavior of *Drosphila melanogaster*[31], could further test the reason for our observed resting preferences.

How does mating status influence female behavior and physiology?

For each mosquito collected, we recorded whether she contained any blood, whether she had developed eggs, and whether she had mated. In doing so, our goal was to test several aspects of *Ae. aegypti* behavior and physiology that were either poorly described in the field or for which ambiguity exists in the literature.

In our study, a similar proportion of virgins (82.1%) and mated females (78.7%) had at least some blood in their abdomen, suggesting that both groups blood feed at similar rates. It had been well-established that virgins blood feed readily in the laboratory, and most laboratory studies have found that virgins are just as likely to

blood feed as mated females[32, 33, 34]. However, to our knowledge only two studies have reported observations that virgins blood feed in a field setting. Teesdale[35] recorded that about 5% of gravid females were unmated in a field study in Kenya, and since *Ae. aegypti* is anautogenous[36], these females must have taken blood meals. He also recorded many unmated females captured via human landing capture and concluded that these mosquitoes were attempting to feed. Pant and Yasuno[37] recorded slightly more females that were blood fed than inseminated in a mark release recapture study in Thailand, suggesting that virgins do feed in the field. However, they only reported the total proportion blood fed and the total proportion inseminated. Therefore, the present study provides robust field evidence that corroborates what many have claimed from laboratory experiments.

Do females always feed before they mate? Teesdale[35] observed that all nulliparous, unfed females (n = 67) collected during a human landing capture were virgin. Because these mosquitoes were assumed to be host-seeking, he concluded that feeding is a prerequisite for mating. To examine this in our study, we quantified the proportions of mosquitoes that were (a) virgin and blood fed, (b) mated and unfed, or (c) both mated and blood fed. We found 20 times more blood fed virgins than mated, unfed females. Superficially, this appears to suggest that females almost always feed first. However, we emphasize that the first action of mated and blood fed females (representing 64% of females in this analysis) remains unknown. It is plausible that blood fed virgins are laden with weight and unlikely to mate quickly after they feed (as mating occurs in flight[38]). On the contrary, females are likely to encounter their mates near the host[38] and thus may have ample opportunity to feed shortly after

mating. Under such a scenario, females that mated first would be underrepresented in our collections. Thus, we refrain from speculating which event came first in females that were already mated and fed at the time of collection. Rather, we simply conclude that neither mating nor feeding first is a requirement in this mosquito population, and that at least 32% of females collected in homes feed prior to mating. Using human landing capture to quantify insemination rates in unfed, nulliparous females (ideally in varied locations) will better illuminate how frequently females mate before they feed.

Virgins that contained blood in our study had abdomens that were, on average, less distended than those of mated females. Because seminal fluid components received during mating induce drastic behavioral[39, 40, 41, 42, 43] and physiological[44, 45, 46, 47] changes in females, one might expect that blood intake varies between mated and blood fed females. Different authors have undertaken laboratory experiments to discern whether virgins or mated females take larger blood meals, and these studies have yielded mixed results[39, 48, 49, 50]. The ambiguity between their findings may be due to small differences in their experimental designs, including the method used to quantify blood or the age at which females fed[49]. Additionally, all three prior studies used restrained Guinea pigs as a blood meal source[39, 48, 49]—an unlikely source for *Ae. aegypti* in an environment with abundant humans.

By measuring engorgement of field-caught females, we have provided evidence in favor of virgins taking smaller blood meals. We accomplished this by developing a new method for assessing engorgement that is scaled to body size. The ratio of inter-sclerite distance:femur width is a rapid, low cost, accurate metric that is ideal for measuring the engorgement of field-caught mosquitoes. However, we note

that our measurement does not directly measure blood meal size, but rather abdominal distension. It is possible that differential rates of diuresis or blood meal digestion, as has been suggested in *Ae. aegypti*[47], could influence distension.

Finally, we have demonstrated that a substantial proportion—more than 20%—of gravid females in the wild are not mated. The presence of virgin, gravid females suggests that finding a mate should not be taken for granted, but rather may limit some females from quickly reproducing. Despite virgins having smaller blood meals overall, gravid virgins contained a similar number of eggs to their mated counterparts. This is the first quantification of egg production in gravid, virgin, field-collected *Ae. aegypti*.

Conclusions

We have demonstrated that in Medellín, female *Ae. aegypti* prefer to rest in bedrooms, consistent with reports from other Latin American settings. However, in contrast to other studies, we show that these mosquitoes often rest above 1.5 m from the floor. Targeting indoor residual spraying to these important locations could save both time and money.

We have also investigated differences in the behavior and physiology of virgin and mated females. We provide conclusive evidence that *Ae. aegypti* frequently blood feeds as virgins, and that neither blood feeding nor mating must occur first. We also find that virgins in the field took significantly smaller blood meals than their mated counterparts—field evidence for a phenomenon that has received mixed support in laboratory studies. Despite this, virgins frequently fully produced eggs in our study.

The fact that a substantial proportion of gravid females had not mated suggests that lack of a mate may prevent some females from quickly laying the eggs they have produced.

In summary, we have provided important insight into the resting behavior and blood feeding behavior and physiology of *Ae. aegypti* in Medellín, Colombia. Our results will benefit efforts to curb arbovirus transmission in this city, and they provide insight into how *Ae. aegypti* bionomics may vary by location. Understanding this vector's biology in epidemiologically relevant settings is imperative to translating laboratory-developed control methods into successful mosquito control strategies in the field.

Illustration 6.2. Home in which 28 *Ae. aegypti* females were collected. Doorway in center of picture leads to the dark bedroom in which 20 were collected from the ceiling and upper portion of the walls. The rest of the home held only 8 female *Ae. aegypti*. No breeding containers were located on the premises.

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CHAPTER 7

RESEARCH SUMMARY AND FUTURE DIRECTIONS

Research summary

The objective of this dissertation was to develop a knowledge base that will assist in future investigations of the molecular and cellular underpinnings of reproductive events in *Ae. aegypti*. To accomplish this, I have (a) laid out a detailed chronology of several reproductive events relative to a female's first mating, including re-mating, sperm modification, oviposition, and fertility, (b) developed comprehensive proteomes of *Ae. aegypti* sperm and seminal fluid, and (c) described resting and feeding behavior in Medellín, Colombia.

While several previous studies had investigated the prevalence of polyandry in *Ae. aegypti* females, disagreement existed as to how frequently it occurs and when it occurs. Williams and Berger[1] and Young and Downe[2] both claimed that polyandry occurred relatively frequently in females that had taken many blood meals and laid many clutches of eggs. By contrast, Helinski et al.[3] described low levels of polyandry that occurred within 48 h of the first mating event. To clarify this discrepancy, I devised a laboratory experiment that quantified the willingness of females to re-mate over a 24 h period after they first mated, as well as after up to five gonotrophic cycles or 30 d after mating. Nearly a quarter of females within 2 hpm accepted a second insemination, whereas this sharply decreased after 16 hpm. By contrast, no females that underwent one, three, or five gonotrophic cycles was re-inseminated. Thus, in this

experiment refractoriness to mating set in within about 24 hpm and was maintained for a female's entire life.

To further refine the timeline of reproductive events, I next described how sperm are modified after insemination. Previous investigations of *Toxorhynchites brevipalpis* and *Culex quinquefasciatus* suggested that sperm lose an outer glycocalyx and its associated plasma membrane sometime after mating[4, 5]. However, these studies did not describe the timing with which such changes occur or whether this is typical of all sperm. Using cryo-electron microscopy, I described the ultrastructure of *Ae. aegypti* sperm in unprecedented detail, documenting novel features such as extranuclear vesicles nestled within the sperm head and a strikingly organized, periodic structure to the glycocalyx. Sperm from the bursa (shortly after insemination) were not morphologically different than those from the male seminal vesicle. However, beginning shortly after mating, the glycocalyx and an outer plasma membrane were removed from sperm. From the spermathecae, no imaged sperm had a fully intact glycocalyx. Rather, this layer was either partially removed or entirely removed. The proportion of fully transformed sperm increased with increasing time post-mating, and by 24 hpm no glycocalyx was observed on any sperm.

In preparing samples for imaging, I noted that sperm in the spermathecae shortly after mating often appeared slower than those from later times post-mating. To quantify motility in the spermathecae over the course of 24 h, I developed an assay in which females were mated, their spermathecae were squashed in saline, and the time required for 20 sperm to emerge from the spermathecae was recorded. Sperm at 2 hpm were extremely sluggish, but 20 sperm exit time increased with increasing time post-

mating. Using this assay, sperm motility plateaued around 8 hpm. In addition, I noted that sperm at early time points appeared more disorganized than later time points, perhaps contributing to their inability to exit the spermathecae quickly.

Given that all of these changes to female mating behavior and sperm physiology occurred within 24 h of insemination, I next tested whether the stimulation to lay eggs also sets in over this same period. I mated gravid females at 2 h intervals for 24 h and then allowed all females a 2 h period to lay eggs. Oviposition was sporadic prior to 12 hpm, but after this time point the number of females laying eggs increased drastically. Given that oviposition is thought to be induced by a seminal fluid protein, and not necessarily the ability to fertilize eggs, I reasoned that females may become fertile before they are stimulated to lay eggs. To test this, I developed an assay to force females to lay their eggs using death stress oviposition. Fertility gradually set in from 4 – 16 hpm, at which time no further increase in fertility was observed. Taken together, females become refractory to a second mating, sperm are modified, females begin laying eggs, and those eggs are able to be fertilized all within 12 – 24 hpm of a female's first mating.

For 50 years, it has been known that mating induces drastic changes to the female physiology and behavior, and the above results have, for the first time, laid out detailed timelines for how quickly many of these changes are initiated. However, the molecular drivers of these changes remain largely unknown. Previous work has suggested that the primary inducers of refractoriness, oviposition behavior, and other physiological or behavioral events is a seminal fluid protein(s) [6, 7]. To assist in the discovery of modulators of female reproductive physiology and behavior, as well as

understand the biology of sperm within the female, I created a comprehensive *Ae. aegypti* "ejaculatome." First, I isolated sperm from the seminal vesicle of over 800 males. Next, I collected the ejaculate of recently inseminated females. These females had themselves been labeled with isotopically heavy nitrogen, but their mates were not; this technique allowed the identification of only male proteins using standard mass spectrometry algorithms. Finally, all of these proteins were subjected to tandem mass spectrometry. In all, 870 sperm proteins and 280 seminal fluid proteins were identified. In parallel, transcriptomes were developed for the major source tissues of these samples (the testes and the male accessory glands, respectively), and the resulting datasets largely corroborated and supported the proteomes. In general, both the sperm and seminal fluid proteomes resembled those of other insects. Interestingly, seminal fluid proteins were over-represented on chromosome 1 of the genome, and sperm proteins on chromosome 2.

In order to translate the findings of laboratory experiments, such as those above, to successful mosquito control in the field, care should be taken to intimately understand the biology of these mosquitoes in a field-relevant setting and with native strains of mosquitoes. To this end, I investigated *Ae. aegypti* resting, feeding, and mating behavior in Medellín, Colombia. I found that homes on the first floor and those with cats were significantly more likely to harbor female *Ae. aegypti*. Within homes, these mosquitoes preferred to rest in bedrooms and on ceilings. While their propensity for bedrooms has been described by other authors, most prior descriptions have shown that females prefer to rest below 1.5 m of height. Because the climate of locations in these studies is substantially warmer than Medellín's, we suspect that mosquitoes in

previous studies may have been avoiding higher than optimal temperatures in thermally stratified rooms.

Our collections also allowed us to probe differences in behavior and physiology between mated and virgin females. Virgins and mated females were equally likely to have blood, suggesting that the two blood feed at similar rates. However, we also showed that virgins take slightly smaller blood meals than their mated counterparts. Despite being less engorged, virgins that were gravid in our experiments produced a similar number of eggs to their mated counterparts. Finally, we observed both blood fed virgins and non-blood bed, nulliparous, mated females, suggesting that neither event must occur first in wild females.

Future directions

What is the impact of mating dynamics on inundative male release strategies?

The primary application of this study to mosquito control efforts is the potential impact of polyandry on mass release strategies. Inundative male releases whose aim is population suppression (such as those that employ sterile[8], genetically-modified[9], or *Wolbachia*-infected males[10, 11]) rely on liberated males competing successfully for wild female mates. Our finding (and those of [12]) that polyandry is likely to occur in the wild may not intrinsically be detrimental to a release strategy, assuming that re-insemination occurs randomly after a female has mated with released or wild males. However, if females preferentially re-mate after mating with a released male, the success of a release may experience diminished results. That is, if a female chooses to re-mate based on some perceived deficiency (or difference) in her released

mate compared with wild males, such a choice may increase the paternity of wild males. Similarly, other mechanisms of cryptic female choice exist (such as sperm dumping or sperm choice after multiple inseminations) may result in similar outcomes. Deeper investigations of *Ae. aegypti* reproductive behavior and physiology should address such questions.

In addition to these basic questions, future work should test whether there are behaviors or mating interactions that are specific to the wild and released strains in locations where inundative releases have taken place. While I have demonstrated that our laboratory strain of mosquitoes (derived from field collected individuals in Thailand several years prior) is only re-inseminated in the short term after mating, it is possible that the phenotype we observed is either strain-specific or due to some form of laboratory adaptation. For example, cuticular hydrocarbons may play a vital role in mating interactions[13, 14], and it is possible that mosquitoes reared on a standardized laboratory diet have a different cuticular hydrocarbon profile than their wild counterparts. Care should be taken to address such questions, using as close to a field-relevant scenario as possible.

What molecules make-up the glycocalyx, and how is it removed?

Little is currently known about the mosquito sperm glycocalyx. Studies of its purpose will likely require background work to better understand its composition. While it is thought to be made of carbohydrate residues[4] deposited by the testes and vas deferentia after sperm have completed individualization[5], defining its composition in greater detail would be beneficial and may be accomplished by mass spectrometry.

Tandem mass spectrometry may also be used to identify the constituent proteins of the outer plasma membrane, some of which likely anchor the glycocalyx to sperm. In addition to understanding glycocalyx composition, defining the physiochemical environment that sperm experience in the bursa and spermathecae may help to design *in vitro* assays whose conditions adequately mimic *in vivo* conditions. Introducing ion- or pH-dependent fluorophores into the reproductive tract via artificial insemination may help to better describe the female reproductive tract's chemistry. These basic characteristics of sperm and reproductive tract biology will bolster experiments that begin to decipher how the glycocalyx interacts with the female reproductive tract and what causes its removal. For example, *in vitro* incubation of sperm in male accessory gland homogenate or a homogenate of spermathecae (from virgins or mated females, as has been done in a grasshopper's sperm[15]) could reveal what conditions or enzymes ultimately cause its removal.

What (if any) is the causal relationship between sperm modification and fertility?

We have shown a tight coordination of reproductive behavior and physiology within the first 24 h of insemination. Reproductive behaviors (such as mating and oviposition) shift at the same time that sperm are completing the shedding of their glycocalyx and females are becoming fertile. While it is tempting to speculate regarding causal relationships among these events, it is premature to draw such conclusions with these experiments alone. Given that seminal fluid is known to initiate many post-mating responses, experimental manipulations that uncouple the receipt of seminal fluid and sperm may help to disentangle the relationships between sperm

modification, oviposition, and fertility. For example, intrathoracic injections of male accessory gland homogenate has been shown to recapitulate much of a female's post-mating response, and artificial insemination[16, 17, 18], surgical removal of accessory glands[19], or genetic ablation of accessory gland function (as was accomplished with *Drosophila melanogaster's* male accessory glands kalb et al 1993-20, chow et al 2015-21[20]) may be a means by which sperm could be delivered to the female without most seminal fluid components.

How are sperm nourished and sustained?

Little is known about how mosquito sperm are nourished. The name "mitochondrial derivative" may imply that mosquito sperm motility is accomplished by energy production in this organelle, but it is questionable whether there is sufficient internal surface area in the mitochondrial derivative to undergo oxidative phosphorylation[22]. In addition, Bao and de Souza[23] described only weak cytochrome C activity in another mosquito's sperm, suggesting that aerobic respiration is not a primary energy source for these cells. On the other hand, the sperm proteome described herein contains a high abundance of both TCA cycle and electron transport chain enzymes. In addition, seminal fluid contains a combination of catabolic enzymes that are well-suited to converting proteins to TCA cycle precursors via gluconeogenesis. Given prior ambiguity surrounding mosquito sperm metabolism and our results, forthcoming experiments will aim to describe exactly how sperm are powered. It is possible that the mechanism sustaining sperm shifts as they move into the spermathecae.

Mining the ejaculatome for critical reproductive proteins and pathways

The primary purpose of creating the *Ae. aegypti* sperm and seminal fluid proteomes was to create a catalog of proteins from which crucial reproductive proteins may be identified. As an example, the presence of a peptide hormone that initiates most of a female's post-mating behavioral changes has been hypothesized for decades[6, 7], although its identity has remained elusive. Experiments to identify this protein(s) are underway, including different methods of fractionation to narrow the list of candidate proteins and developing CRISPR knock-outs to test the function of individual proteins[24]. Ultimately, leveraging this dataset to identify this (or other reproductive) protein(s) will illuminate molecular targets that may be manipulated for the purposes of vector control.

Applying mosquito behavioral observations in Medellin to vector control strategies

We aimed to understand *Ae. aegypti* in the field in a dengue endemic setting so that mosquito control efforts could be tailored towards the local mosquitoes' biology. Our finding that *Ae. aegypti* in Medellín prefers to rest high on walls and on ceilings differs from the results of other studies[25,26,27], and future work should identify whether this is an environmental effect (e.g., due to temperature in the room) or whether this is a trait specific to the *Ae. aegypti* strain in Medellín. If an indoor residual spray campaign were to be implemented in Colombia, it may be interesting to test whether the selective application of insecticides (e.g., to ceilings only) is as effective as comprehensive coverage of all potential resting surfaces. A similar comparison of

spraying regimes in Mexico suggested that substantial cost could be saved by only treating surfaces on which *Ae. aegypti* is most likely to rest[28].

Comparing bionomics of females with and without Wolbachia *in Medellín*

The World Mosquito Program aims to replace the Medellín population of *Ae. aegypti* with mosquitoes that harbor *Wolbachia* endosymbionts, in an attempt to make wild mosquitoes resistant to dengue and other arboviruses[29]. At the time of our collections, only pilot scale releases had taken place in Medellín, and these releases were not successful in establishing a stable, fixed population of *Wolbachia*-positive mosquitoes (Simon Kutcher, personal communication). Future investigations of *Ae. aegypti* in Medellín should investigate potential differences in the bionomics of *Wolbachia*-positive and –negative mosquitoes. Mating interactions between these two groups should also be investigated, as well as between laboratory-reared and wild mosquitoes. Such studies may pre-emptively identify barriers to *Wolbachia's* successful invasion of the Medellín population of *Ae. aegypti* and are important to conduct prior to full-scale releases of modified mosquitoes.

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