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Targeting Insect Reproduction as a Control Mechanism: Sperm Movement From the Seminal Vesicles to the Egg

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The text of this dissertation, Chapter 5 in full, is a reprint of the material as it appears in Insect Biochemistry and Molecular Biology, 2015. Dr. Richard Cardullo and Dr. Catherine Thaler listed in that publication supervised the research.

DEDICATION

I would like to dedicate my dissertation work to my sister, Kerri Stephens. She has been my best friend for as long as I can remember. She has inspired me and encouraged me throughout the entire process.

ABSTRACT OF THE DISSERTATION

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by

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Mosquito sperm are produced in the testis, transferred to the seminal vesicles and subsequently transferred along with accessory gland fluid to the female. Sperm from the water strider, *Aquarius remigis*, are unusually long, possessing a complex acrosome and flagellum. In order to be fertilization competent, sperm in both species must undergo several developmental steps in both the male and female reproductive tract before the sperm is competent to fertilize an egg. Once the sperm reaches the egg, it must have a mechanism for interacting with the egg. In this dissertation, I follow the sperm from the testis, the site of spermiogenesis, to the egg where it interacts with the sperm. In the second chapter, I explore the role of muscle contractions of the seminal vesicles and accessory glands that likely move sperm and accessory gland fluid from the male to the female. Nicotine and acetylcholine both activate muscle contractions but not pilocarpine, octopamine, serotonin indicating that muscle contractions are activated by a nicotinic acetylcholine receptor. In the third chapter, I identify and characterize trypsins from accessory glands of *Culex pipiens* that are necessary for sperm motility initiation. I show

activity levels and pH optima of accessory gland trypsins and identify several potential trypsin-like proteins that could be involved in activating sperm motility. In the fourth chapter, I characterize glycosidases on *Aquarius remigis* sperm. These glycosidases are potentially involved in recognizing glycosylation that is present on the egg surface near the micropyle. In the fifth chapter, I explore the possibility of circadian rhythms being involved in timing the movement of sperm from the testes to the seminal vesicles. Three genes: *ssr*, *npy* and *5ht*, are expressed in the testes and are commonly involved in regulating circadian rhythms in other organisms. In this dissertation, I discuss movement of sperm from the testes to the seminal vesicles via circadian rhythms, movement of sperm from seminal vesicles to the female via muscle contractions, activation of the sperm motility, allowing it to reach the egg, and recognition of the egg via glycosylation. Taken together, the chapters in this dissertation provide insight into movement of sperm from the testes to the female reproductive tract.

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CHAPTER 1

LITERATURE REVIEW

Targeting Insect Reproduction as a Control Mechanism: Sperm Movement from the Seminal Vesicles to the Egg.

Introduction

In animals, successful fertilization occurs when two haploid cells, the egg and the sperm, fuse to produce a diploid zygote. Gametes are a unique cell type in that they are exposed to several different environments during their development and must be able to respond to environmental changes. In order to participate in fertilization, gametes must undergo requisite developmental steps, including sperm acquisition of motility, membrane reorganization, environmental factors and post-translational modifications to prepare for fertilization (Baker et al., 1968).

The sperm is usually composed of two specialized cellular regions: the head and the flagellum. In many animals, including most vertebrates, the head contains only two organelles, the haploid nucleus and the acrosome. In mammals, this secretory organelle releases its contents, including enzymes that aid in the penetration of the extracellular egg coat. The flagellum, which may or may not contain functioning mitochondria, propels sperm toward the egg after motility has been activated. Depending on the organism, various factors including dilution, compounds or peptides secreted by the egg, and changes in the environment can activate motility. Once the gametes have undergone final requisite maturation processes in the female reproductive tract, the sperm travels to the oocyte and subsequently recognizes, binds, and ultimately fuses with the egg resulting in successful fertilization.

The molecular mechanisms involving sperm-egg recognition and sperm motility regulation have been extensively studied in non-insect systems (Downey and Lambert, 1994; Habermann and Sinowatz, 2011; Sinowatz et al., 1998; Focarelli et al., 2001).

These studies are reviewed in this chapter and, when pertinent, comparisons are made between non-insect systems and insect systems. I will further describe what is known about reproduction in two insect systems: water striders and mosquitoes as these organisms are the basis of my studies.

Insect Population Control Mechanisms

Mosquitoes are important vectors of many diseases that cause extensive morbidity and mortality. According to the World Health Organization (WHO), insect transmitted diseases are present in more than 100 countries and are most prevalent in developing countries. Despite progress being made with respect to controlling insect populations, the number of people becoming ill with arboviruses (viruses transmitted by arthropod vectors) is still too high. Arboviruses account for one sixth of the infections associated with Disability Adjusted Life Years (DALYs) in the world and 90% of these cases are mosquito transmitted (McGraw and O'Neill, 2013). The prevalence of these diseases affects communities in numerous social and economic ways. For example, those that cannot work because they have an insect transmitted disease can be an economic burden for their community. The pathogens that mosquitoes carry often cannot be transmitted vertebrate to vertebrate and, therefore, rely on the mosquito to transmit them. This requirement suggests that mosquitoes are a suitable target in preventing disease transmission and reducing morbidity and mortality.

There are four main ways to control mosquito populations (behavorial, insecticidal, genetic and biological), but none of these methods are a panacea. Controlling

mosquito populations is of utmost importance; however, as with other insects, mosquitoes are developing resistance to traditional insecticides (Ransin et al., 2011; Asidi et al., 2012; Wondji et al., 2012). Many *Culex* mosquitoes have already developed pyrethroid resistance (Corbel et al., 2007; Li and Liu, 2010). As mosquitoes develop resistance to insecticides, it will become imperative to understand multiple aspects of their life history, in order to identify new targets for population control. Understanding the unique biology of mosquitoes allows for the development of novel insecticides to target mosquito reproduction and prevent disease.

Life History and Fertilization in the mosquito, *Culex pipiens*

Culex is a genus of mosquitoes that includes species ranging from 4 to 10 mm long. They are vectors for several important diseases such as West Nile Virus, Japanese Encephalitis Virus, St. Louis Encephalitis Virus and filariasis (Vinogradova, 2000). They exist in the Northern United States, Europe, Asia, Africa and Australia (Vinogradova, 2000) where their larvae develop in standing water. Adult females might take a blood meal to aid in egg development. Some *C. pipiens* are, however, autogenous, meaning that they do not require a blood meal for egg development (Vinogradova, 2000). Male mosquitoes become sexually active 2-3 days post emergence as adults (Vinogradova, 2000) and fly in a swarm and initiate a mating sequence when a female comes within range of the swarm (Gibson, 1985). The number of swarming individuals increases rapidly in the first 5-10 minutes and reaches numbers of several thousand individuals (Vinogradova, 2000). The mosquitoes can swarm for 1.5-2 hours with mating starting 10-

20 minutes after the swarm forms (Vinogradova, 2000) and lasting up to 45 minutes (Vinogradova, 2000). The male mosquito uses the Johnston's organ, a specialized organ located on its antennae, to locate the female mosquitoes in a species-specific manner (Vinogradova, 2000). The Johnstons organ perceives the tone produced from the female's wingbeat frequency (Vinogradova, 2000), which is 100-200/sec lower than that of the male mosquito (Vinogradova, 2000).

After mating and transfer of sperm to the female, motility will be activated. Activation of sperm motility in a closely related species, *Culex quinquefasciatus*, can be stimulated by a trypsin-like protease. A plasma membrane associated ion channel has been implicated that allows an influx of extracellular calcium, which is required for motility activation (Thaler et al., 2014). Unpublished data indicates that *C. pipiens* motility can also be activated by trypsin. Specifically, *C. quinquefasciatus* display a unique motility pattern (Thaler et al., 2014) that has three separate and distinctive waveforms (Figure 1A/B). Wave A consists of a low amplitude, long wavelength wave; wave B consists of a double helical wave in which a low amplitude, short wavelength wave is superimposed upon a high amplitude, long wavelength wave; wave C is a single helical wave (Figure 1A/B/E; Thaler et al., 2014). MAPK-directed phosphorylation is involved in regulating these waveforms (Thaler et al., 2014). Unlike non-insect sperm, *C. quinquefasciatus* sperm exhibit backwards motility, which is regulated by $[Ca^{2+}]_i$ (Thaler et al., 2014). In backwards motility, the wave is propagated from the tail tip towards the sperm head. *C. quinquefasciatus* sperm swim backwards in conditions of low $[Ca^{2+}]_i$ and increased phosphorylation. *Culex* are not the only Dipterans (Drosophila, Tephritid and

Phorid) that exhibit backwards motility either *in vitro* or *in vivo* (Bacetti et al., 1989; Curtis and Benner, 1991; Kottgen et al., 2011; Yang et al., 2011; Yang and Lu, 2011).

Life History and Fertilization in the water strider, *Aquarius remigis*

Aquarius remigis is an abundant semiaquatic insect found throughout North America that feeds on insects trapped on the water surface. Although much is known about *A. remigis* mating behavior, little is known about the molecular mechanisms that result in fertilization. In order to mate, the male water strider attempts to mount the female and the female resists this attempt (Weigensberg and Fairbairn, 1994; Sih and Watters, 2005; Arnqvist, 1997). Male water striders use aggressive behaviors to grasp, mount, and ultimately force copulation, which under laboratory conditions, can last for more than 12 hours (Weigensberg and Fairbairn, 1994; Arnqvist, 1997). The copulatory event is substantially longer than the time required for insemination (Rubenstein, 1989), which occurs near the end of copulation (Campbell and Fairbairn, 2001). The sperm are released from the seminal vesicles, deposited in the long and labyrinthine gynatrial complex, are transported through the vermiform appendix to the spermathecal tube where sperm can be stored for long periods of time (Campbell and Fairbairn, 2001). In some cases, sperm go directly to the fecundation canal where they can fertilize an egg (Campbell and Fairbairn, 2001). It is possible that the complex architecture of the female reproductive tract influences male fertilization success and that it has a selective influence on the selection of sperm form. In other insects, there is evidence that the female reproductive tract influences the evolution of sperm morphology (Higginson et

al., 2012; Presgraves et al., 1999; Miller and Pitnick, 2002). In water striders, the ~5 mm long sperm must traverse a 6 mm long, narrow and winding fecundation canal (Jamieson, 1987) before fertilization can occur. The acrosome is long, has a rigid portion and may play a role in moving through the fecundation canal's distal end (Campbell and Fairbairn, 2001; Miyata et al., 2011). The rigidity in part of the acrosome may be due to tubules that extend along the length of the acrosome (Tandler and Moriber, 1966) and are likely composed of an acrosomal matrix protein (Miyata et al., 2011). It remains unknown how the entire ~5 mm long sperm, possessing a 2.5 mm long acrosome and a 2.5 mm long flagellum, enters an egg that has a diameter of 1.0 mm along its long axis (Figure 1C/D; Miyata et al., 2011). The flagellum is structurally similar to other insect species with a "9+9+2" microtubule based axoneme (Figure 1F) and a modified mitochondrial sheath surrounds the microtubules (Tandler and Moriber, 1966).

Despite not being a medically or economically relevant species, the structure of the water strider sperm makes it an optimal study system for sperm egg-recognition studies. The large sperm of *A. remigis* are optimal for biochemical studies as copious amounts of protein can be extracted and studied. The sperm-egg interaction is likely to occur on the plasma membrane overlying the acrosomal region of the sperm (Evans, 2012). But, in most organisms, the acrosome makes up a minor fraction of the sperm volume and surface area and, thus, acrosomal proteins, and plasma membrane proteins surrounding the head, would make up only a small percentage of the total sperm plasma membrane proteins. In contrast, in *A. remigis*, round acroblasts develop into long acrosomes (Ott et al., 2015) making up approximately half of the sperm's surface area

suggesting that if the receptor for the egg is found along the entire length of the acrosome, there will be a higher proportion of the receptor in whole sperm. Based on images from Miyata et al. 2011, the surface area of the water strider acrosome is approximately 30,000 μm^2. In comparison the surface areas of acrosomes of *Drosophila melanogaster*, mouse, human and boar are 33 μm^2 (Jamieson, 1987), 20 μm^2 (Baltz and Cardullo, 1989), 10.5 μm^2 (van der Horst and Maree, 2009), and 34.45 μm^2 (van der Horst and Maree, 2009), respectively. This large difference in acrosomal surface area could allow for easier biochemical studies of the proteins overlying the water strider sperm acrosome that are involved in sperm-egg interactions if the receptor is uniformly distributed over the plasma membrane.

Circadian Rhythms and Sperm Movement from the Testes

Circadian rhythms are endogenous biological rhythms persisting under constant environmental conditions with a 24-hour period length (Kennaway, 2005). Daily rhythms controlled by intrinsic clocks are common within the animal kingdom. In mammals, the central clock is located in the suprachiasmatic nucleus (SCN) of the brain and is based on regulated transcription and translation of clock proteins. Other neurotransmitters and their cognate receptors that are known to be involved in circadian rhythms include neuropeptide Y, serotonin and somatostatin (Kennaway, 2005). The expression of *period* (*per*) and *cryptochrome* (*cry*) genes results in the inhibition of *clock* and *brain and muscle arnt-like protein-1* (*Bmal1*) genes, which in turn causes the expression of *per* and *cry* genes. Further, when PER and CRY have high expression, they interact to repress

their own transcription (van der Speck et al., 1996; Kobayashi et al., 1998; Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Takumi et al., 1998; Takumi et al., 1998; Tei et al., 1997; Zylka et al., 1998).

In many organisms, circadian rhythms influence reproduction. Cyclic expression of circadian genes within the peripheral tissues is well established. In mice, circadian genes are cyclically expressed in peripheral tissues (Kobayashi et al., 1998; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997; Zylka et al., 1998; Zylka et al., 1998; Sangoram et al., 1998; King et al., 1997), which can be decoupled from SCN expression (Balsalobre et al., 2000; Damiola et al., 2000; Stokkan et al., 2001). For example, the oviduct expresses the clock genes in a rhythmic fashion (Kennaway, 2005). Insect reproductive tissues express CLOCK proteins cyclically, which can control sperm release (Beaver et al., 2002). Drosophila displays decreased fertility when the circadian genes are mutated (Beaver et al., 2002). If a circadian clock functions in the testis, clock genes should be expressed in the tissues and could potentially be cyclic. In most mammals, circadian rhythm gene expression is not cyclic (Morse et al., 2003) but in hamsters, the clock gene is rhythmic in the testis (Tong et al., 2004). *Period1* (*per1*) expression increases during later stages of sperm development (Morse et al., 2003). There is a change in *per1* levels over the course of sperm maturation suggesting a clock may be involved in regulating spermatogenesis (Alvarez et al., 2003; Bittman et al., 2003). *Bmal1* mutants have impaired fertility and a high incidence of abnormal sperm (Kennaway, 2005). In Lepidopterans, the pupal testis contains mature sperm as spermatogenesis starts in the late larval stages (Giebultowicz et al., 1988). Several

species of Lepidopterans release sperm in a rhythmic fashion including: *Anagasta kuehnilla* (Thorson and Riemann, 1977), *Pectinophora gossypiella* (LaChance et al., 1977) and *Lymantria dispar* (Giebultowicz et al., 1988). It is unknown if sperm movement from testis to seminal vesicles is rhythmic in other non-Lepidopteran insects.

Muscle Contractions and Movement of Seminal Fluid from the Seminal Vesicles and Accessory Glands

In mammals, the smooth musculature of seminal vesicles receives both adrenergic innervation (Falck et al., 1965; Sjostrand, 1965) and cholinergic innervation (Waddel, 1917; Bacq, 1931; Eliasson and Risley, 1966; Al-Zuhair et al., 1976). The seminal vesicle muscles can be stimulated to contract *in vivo* by directly stimulating the nerves (Karr et al., 1973). The α/β-adrenergic agonist adrenaline, the α1-adrenergic agonist phenylephrine, and the cholinergic agonist acetylcholine, but not the β-adrenergic agonist terbutaline, the α- adrenergic antagonist phentolamine, the β-adrenergic antagonist propranolol, or the anticholinergic compound atropine, can induce muscle contractions in rat seminal vesicles (Hib et al., 1984). Phentolamine but not propranolol blocks atropine and phenylephrine induced contractions (Hib et al., 1984). A muscarinic acetylcholine receptor was indicated based on the antagonistic effects of atropine (Hib et al., 1984). Inhibiting contractions of the seminal vesicle is the major focus for treating premature ejaculation, which is second only to erectile dysfunction as a common sexual disorder (Birowo et al., 2010; Birowo et al., 2010; Hsieh et al., 2010). Despite this, only a few studies have been published on the mechanism and regulation of seminal vesicle muscle

contractions including the effects of phosphodiesterase inhibitors and 5-hydroxytryptamine 1A activators (Birowo et al., 2010; Hsieh et al., 2010).

Ejaculation is modulated by a complex interplay of neurotransmitters including dopamine, serotonin and oxytocin (Giuliano, 2007). 5-hydroxytryptamine (serotonin) is the most widely studied and pertinent neurotransmitter involved in ejaculation (Giuliano, 2007). 5-HT_{1A}, 5-HT_{1B} and 5-HT_{2C} receptors are involved in ejaculation and found within the seminal vesicles and the vas deferens (Giuliano, 2007). 5-hydroxytryptamine inhibits ejaculation by activating inhibitory efferent pathways (Giuliano, 2007). Increasing serotonergic neurotransmission reduces premature ejactulation (Giuliano, 2007). Nojimoto et al., (2009) investigated the effect of sibutramine, a nonselective inhibitor of neuronal reuptake of norephenphrine and serotonin, in vas deferens contractions and found that it increased muscle contractions in the vas deferens but not the seminal vesicles (Figure 2).

The muscle contractions of female reproductive tracts of insects are better studied than the male reproductive tracts. Locusts (Clark and Lange, 2003; Zdarek et al., 2000; Veelaert et al., 1996; Lange and Tsang, 1993; Nykamp and Lange, 2000), stable flies (Cook and Wagner, 1992) and cockroaches (Woodhead et al., 2003) all experience muscle contractions within the female reproductive tract. For example, the ovarioles, oviduct, and peritoneal sheath are all muscular components of the female reproductive tract. These muscle contractions move the egg down the ovarioles (via the oviduct) to the uterus and then through oviposition (Lee et al., 2003; Monastirioti, 2003). The Drosophila uterine wall contains striated muscle (Middleton et al., 2006). Further,

proteins from the seminal fluid can act to stimulate uterine contractions (Avila and Wolfner, 2009).

In male crickets, proctolin causes muscle contractions of the male accessory glands (Kimura et al., 1989). Proctolin and FGLamide-related allostatins (FGLa/AST) cause a change in muscle contractions in the male reproductive tract of *Euborellia annulipes* (Rankin et al., 2009). Contractions in the *E. annulipes* male reproductive tract is thought to be involved in moving seminal fluid through the male reproductive tract (Rankin et al., 2009). Crickets also experience male reproductive tract contractions, specifically in the accessory glands (Kimura et al., 1989).

Serotonin has been implicated in oviductal and spermathecal contractions in insects. Serotonin acts on the oviduct muscles of *Tabanus sulcifrons* (Cook and Meola, 1978), *Periplaneta Americana* (Stoya and Penzlin, 1988; Bamji and Orchard, 1995) and serotonin innervates the spermatheca (Clark and Lange, 2002). The receptors on the oviduct that are involved in the oviductal contractions are 5HT receptors (Lange, 2003).

Sperm Structure and Diversity

In most animals, sperm, unlike oocytes, have evolved to be the small, motile gametes that are produced in large numbers. Most flagellated animal sperm are built on the same general ground plan. In vertebrates, the fully mature sperm consists of three regions: the head that is involved in sperm-egg interaction, the midpiece that is involved in aerobic energy production and contains mitochondria, and the flagellum that generates motility. Insect sperm are similar to that of vertebrates having a head and a long

flagellum for motility. However, the ultrastructure of animal sperm can be highly variable (Bacetti and Afzelius, 1976). Insects exhibit some of the more extreme variations (Bacetti and Afzelius, 1976). Insect sperm can be lacking any of the four main sperm constituents: acrosome, nucleus, flagellum and mitochondria (Jamieson et al., 1999). The adaptations that sperm have are likely to aid in enabling the sperm to perform the many tasks that it needs to in order to successfully complete fertilization.

Extreme examples of flagellar diversity include Proturans, which are aflagellate and Mastotermitidae, which have individuals with ~100 weakly motile flagella (Jamieson, 1987). However, flagellar number is only one way in which insect sperm vary and insect sperm often exhibit dimorphism (apyrene v. eupyrene sperm), as well as differences in sperm size, axonemal structure and conjugation systems. For example, *Asphondylia ruebsaameni* sperm have ~2,500 doublets in their axonemes (Mencarelli et al., 2000). Another common adaptation in sperm is the lack of an acrosome in many species that possess micropyles (Bacetti, 1979). Despite these seemingly extreme modifications, sperm are adapted for one common function: fertilizing an egg. After production in the testis, sperm are still not competent to fertilize an egg and must undergo several complex molecular changes in order to recognize, bind, and fuse with the egg. In addition, in many species, the sperm undergoes many sequential plasma membrane surface modifications as it passes through the male and female reproductive tracts (Yanagimachi, 1994).

Flagellum Structure

The flagellum provides the mechanism for propulsion. The flagellum contains the axoneme, which is a microtubule-based cytoskeletal structure that generates motility. The canonical axoneme has a 9+2 arrangement that contains nine outer doublet microtubules and one central pair. Dynein arms are associated with the outer doublet microtubules. In insects, the sperm length can range from 12 μm in Hymenopterans (Quicke et al., 1992) to 6 cm in *Drosophila bifurca* (Pitnick et al., 1995). Frequently most of this length is the flagellum, which consists of a 9+9+2 axoneme (Werner and Simmons, 2008). Microtubule sliding as dynein arms of one microtubule grip the adjacent pair, pull, and release and bind again causes the axoneme to bend (Afzelius, 1959; Satir, 1965; Satir, 1968).

The two-mitochondrial derivatives present in insect sperm are the product of a complex fusion and separation process that occurs in early spermatid development (Pratt, 1968). There are three motility patterns that vary based on the structure of the axoneme: (1) a simple, planar, low frequency, long wavelength flagellar wave observed in insects with a 9+2 axonemal structure, (2) a 3D helical wave observed in insects with a 9+9+2 axonemal structure and (3) a double helical wave pattern with superimposed flagellar waves in insects with ATPase activity endowed accessory bodies and a 9+9+2 axonemal structure (Bacetti, 1972). The third motility pattern has been described in several insect species including *Tenebrio molitor* and *Bacillus rossius* (Bacetti, 1972), *Lygaeus sp*. (Phillips, 1974), *Culicoides melleus* (Linley, 1979), *Aedes notoscriptus* (Swan, 1981), *Ceratitis capitata* (Bacetti et al., 1989), *Drosophila sp.* (Bressac et al., 1991), *Megaselia*

scalaris (Curtis and Benner, 1991), *Culex quinquefasciatus* (Thaler et al., 2014) and *Aleochara* (Werner et al., 2000, 2001, 2002). Several insect species also exhibit backwards motility including *Culex quinquefasciatus* (Thaler et al., 2014), *Drosophila* (Kottgen et al. 2011; Yang et al., 2011; Yang and Lu, 2011), *Pediculus humanus* (Ito, 1966), tephritid flies (Baccetti et al., 1989), *Megaselia scalaris* (Curtis and Benner, 1991), and *Aleochara bilineata* (Werner et al., 2000). The biological function of backwards motility is unclear but one hypothesis is that it aids in increasing maneuverability within the small confines of the female reproductive tract (Baccetti et al., 1989; Curtis and Benner, 1991). It is also possible that the sperm move tail first toward the egg as *Musca domestica* sperm have been observed to enter the egg tail first (Degrufillier and Leopold, 1976).

Motility Regulation

In order to conserve energy sperm undergo a period of quiescence while in the testes (Aitken and Fisher, 2005). Sperm motility in echinoderms and mammals is activated by extracellular cues, which stimulates phosphorylation changes via kinases and phosphatases (Visconti and Kopf, 1998; Darszon et al., 1999; Neill and Vacquier, 2004). Changes in phosphorylation lead to motility activation (Visconti and Kopf, 1998; Darszon et al., 1999; Neill and Vacquier, 2004). Sperm can have different motility patterns in different portions of the reproductive tract and, thus, the engine that controls this motility may need to be turned on or off depending on the circumstances.

One mechanism mammalian sperm use to maintain quiescence in the epididymis is maintaining a low epididymal pH (Levine and Kelly, 1978; Acott and Carr, 1984; Carr et al., 1985). A V-ATPase in epididymal epithelial cells helps maintain a low pH in the epididymal lumen (Shum et al., 2009). A voltage gated proton channel, hv1, elevates pH post ejaculation (Lishko et al., 2010). Mammalian sperm undergo a high amplitude and asymmetric flagellar beating pattern upon entrance into the female reproductive tract known as hyperactivation (Yanagimachi, 1994). This change in beating pattern occurs due to activation of a soluble adenylyl cyclase (SACY) by bicarbonate (HCO_3^-), which activates cAMP signaling and protein tyrosine phosphorylation (Chen et al., 2000; Visconti et al., 1995).

In sea urchins, immotile sperm are spawned directly into seawater (SW) where they are activated. In the testes, a high CO_2 concentration keeps sperm at pH_i ~7.2. When the sperm are spawned into SW the CO_2 tension decreases causing an increase in pH to ~7.6 (Johnson et al., 1983). This increase in pH is sufficient to activate the motor protein that is responsible for microtubule sliding, the dynein ATPase. The excess production of ADP from the catalytic cycle of dynein ATPase results in an increase in cellular respiration (Christen et al., 1982). Speract, an egg jelly decapeptide, activates motility by binding a sperm membrane receptor (Cardullo et al., 1991) and subsequently activates a guanylate cyclase (GC) (Dangott and Garbers, 1984; Bentley et al., 1988). The activation of GC causes many physiological changes including changes in ion concentrations (Darszon et al., 1999; Neill and Vacquier, 2004).

Insect sperm, unlike sperm from other organisms, are motile over a wide pH range. The sandfly sperm, *Culicoides melleus*, is motile between pH 6.1-12.3 (Linley, 1979); whereas Saturniids, *Antherea pernyi* and *Hyalophora cecropia*, are mostly motile at pH 5.8-7.8 (Shepherd, 1974). Often times, sperm are immotile while in the male reproductive tract and only become motile upon ejaculation into the female with seminal secretions (Bishop, 1920; Davis, 1965; Hughes and Davey, 1969; Landa, 1960; Omura, 1936). Seminal secretions have been demonstrated to be necessary for artificial insemination (Omura, 1936; Omura, 1938). Seminal gland secretions from *Stilophilus granarius* (Khan and Musgrave, 1969), and *Melolontha melolontha* have been shown to activate sperm motility (Landa, 1960; Landa, 1961).

In *Cimex lectularias* and *C. hemipterus*, sperm motility requires sufficient oxygen levels (Rao and Davis, 1969) while *Rhodnius prolixus* has pH dependent motility activation (Davey, 1965). Spermathecal gland proteins from female *Apis mellifera* (Koeniger, 1970) and *Anthonomis grandis* (Villavaso, 1975) can also activate motility. In other insects, male accessory gland secretions contain trypsin-like enzymes that can activate motility. A trypsin-like enzyme, initiatorin, secreted from the prostatic gland of *Bombyx mori* activates motility in apyrene sperm. Similarly, Orthopterans also have trypsin initiated sperm motility (Osanai and Bacetti, 1993). The presence of motility activation by trypsin in Lepidopterans and Orthopterans suggests that the process may be conserved in insect species. In the water strider, *A. remigis*, sperm is activated in vitro by trypsin and a Protease Activated Receptor 2 (PAR2) is localized to the flagellum of the sperm (Miyata et al., 2012). Serine proteases can act as signaling molecules by cleaving

PARs. PARs have a conserved mechanism of proteolytic activation. Namely, a protease cleaves at a conserved region in the extracellular N-terminus. Upon cleavage, a tethered ligand domain is exposed and binds a region in extracellular loop two (Cottrell et al., 2003). PAR2 belongs to a family of G-protein coupled receptors that activate the MAPK pathway upon being cleaved by trypsin (Ramachandran et al., 2009) indicating that it is likely that motility is activated by trypsin cleavage of a PAR2-like protein present on the flagellum of *A. remigis* sperm (Figure 3).

Sperm Surface Proteins

The sperm plasma membrane likely undergoes extensive remodeling as it moves through the various environments (Jones, 1989; Yanagimachi, 1994). In order to understand the events that lead up to fertilization, it is very important to understand the cell surface proteins that are present on the sperm surface. From the outset of sperm formation in the testis, sperm are prepared to make any necessary adjustments for motility and sperm-egg interactions since transcription and translation are completely silenced in mature sperm (Boerke et al., 2007). The change in milieu of the sperm cell as it transits from testis to oviduct is likely to affect the composition and organization of sperm plasma membrane proteins. The changes that occur because of the change in environment are likely to alter the sperm's ability to fertilize a mature egg. Proteins on the mammalian sperm surface can diffuse laterally but they are confined to certain regions of the sperm surface (Gadella et al., 1995; Phelps et al., 1988). During transit through the epididymis, the mammalian sperm surface is remodeled via release,

modification and adsorption of proteins and lipids (Sharma et al., 2004; Sullivan et al., 2007). Post-ejaculation, sperm are transported through the uterus where further modifications will occur, and, at this point, sperm become capable of fertilizing an egg. The mammalian sperm undergo capacitation and subsequently become hyperactivated and undergo a number of surface rearrangements.

Little is known about the maturational changes that occur in insects as the sperm transits through the various regions of the male and female reproductive tracts. In insects, there is evidence that the housefly *Musca* undergo capacitation but there is no evidence that hyperactivation occurs (Leopold and Degrugillier, 1973).

One stage that is likely to involve maturational stages in insects is the period of storage in the female spermatheca. Female sperm storage is nearly ubiquitous in insect species (Gillott, 1988; Neubaum and Wolfner, 1999). Fluorescence-lifetime methodology used to monitor cell metabolic rate and ROS production simultaneously allowed researchers to watch ROS production in real-time in *Gryllus bimaculatus* sperm and showed that both metabolism and ROS production are reduced simultaneously (Ribou and Reinhardt, 2012). Sperm storage in the female consists of sperm accumulation, maintenance/retention and seminal vesicle/spermathecal exit but the processes involved are not well understood (Bloch Qazi et al., 2003). Storage can be influenced by muscle contractions, location of ejaculate deposition and sperm motility (Bloch Qazi et al., 1998; Bloch Qazi et al., 2003).

Sperm-egg interactions (via non-enzymatic proteins)

In mammals, a porous extracellular matrix known as the zona pellucida surrounds the oocyte (Dunbar et al., 1994; Wassarman and Litscher, 2008). The sperm bind the ZP and undergo a regulated exocytotic event referred to as the acrosome reaction (Bleil and Wassarman, 1983). Primary binding begins with a loose, non-species specific binding and is rapidly followed by a species-specific tight irreversible binding (Sacco et al., 1989; Bleil and Wassarman, 1983; Inoue and Wolf, 1975). ZP3 is the bioactive component responsible for sperm binding O-linked glycoproteins on the egg surface (Florman and Wassarman, 1985; Litscher et al., 1995). A plethora of candidates for sperm receptors for the egg have been suggested. Some of these include enzymes like β-1,4-galactosyltransferase (Shur and Hall, 1982), fucosyltransferase 5 (Chiu et al., 2007), α-D-mannosidase (Tulsiani et al., 1989), and arylsulfatase A (Hess et al., 1996). These enzymes that are putative receptors will be discussed in more detail later. Here, I will discuss the non-enzymatic putative receptors for the egg. One non-enzymatic gamete receptor on the sperm is SED1 (Stubbs et al., 1990). SED-null mice are subfertile presumably because sperm are unable to bind the ZP despite there being no other obvious defects (Ensslin and Shur, 2003). ZP3R/sp56 was identified as a receptor on sperm based on photoaffinity crosslinking studies (Bleil and Wassarman, 1990; Cheng et al., 1994). ZP3R has a highly specific affinity for O-linked oligosaccharides present on the ZP and preincubation with antibodies directed against ZP3R suppresses in vitro binding to the ZP (Suzuki-Toyota et al., 1995; Hardy et al., 2004). However, immunoelectron microscopy has shown that ZP3R is enclosed within the acrosomal matrix of uncapacitated sperm

(Foster et al., 2003; Kim et al., 2001) making it unlikely to play a role in primary sperm-ZP binding. There are also D-mannose binding sites on the sperm surface (Tesarik et al., 1991) and the presence of these has been correlated with fertilizing ability and ZP affinity (Benoff et al., 1993a; Benoff et al., 1993b, Tesarik et al., 1991). Also, preincubation of sperm with D-mannose decreases sperm affinity for ZP (Rosano et al., 2007). Some other molecules that may be involved in sperm-zona interactions are calmegin (Ikawa et al., 2001; Yamagata et al., 2002), fertilization antigen 1 (Naz et al., 1984; Naz et al., 1992), proacrosin (Urch and Patel, 1991; Baba et al., 1994a; Baba et al., 1994b), sperm adhesion molecule 1 (Hunnicutt et al., 1996a; Hunnicutt et al., 1996b) and sperm autoantigenic protein 17 (Yamasaki et al., 1995).

Nonmammalian eggs are covered with a vitelline envelope and jelly layer. In abalones, sperm lysin recognizes a vitelline envelope receptor for lysin (VERL) (Swanson and Vacquier, 1995). VERL is large glycoprotein (Swanson and Vacquier, 1995). Sea urchin fertilization was predicted to involve a receptor-ligand interaction based on studies that showed that when eggs are saturated with high concentrations of sperm the entire egg surface was not fully covered by the sperm (Vacquier and Payne, 1973) implying a limited number of binding sites. Subsequent studies showed that sea urchin sperm have a receptor (bindin) that recognizes a complementary ligand on the egg (Glabe and Vacquier, 1978). The ligand for bindin, EBR1 protein, is a sulfated fucose polysaccharide (Kamei and Glabe, 2003).

Sperm-Egg Interactions (via enzymes)

Despite a lack of data on the receptor-ligand pairs involved in sperm-egg binding, there is evidence that indicates that the sperm head plasma membrane contains lectin-like molecules that recognize conjugate sugars present on the egg surface (Downey and Lambert, 1994; Habermann and Sinowatz, 2011; Sinowatz et al., 1998; Focarelli et al., 2001). One hypothesis is that enzymes such as glycosidases and glycosyltransferases functioning as lectins may be involved in the primary recognition event between sperm and egg (Mengerink and Vacquier, 2001; Sinowatz et al., 1998; Tian et al., 1997; Downey and Lambert, 1994; Matsumoto et al., 2002; Tulsiani et al., 1997; Tulsiani et al., 1989; Zitta et al., 2006; Shur et al., 2006; Lopez et al., 1985; Lopez and Shur, 1987; Shur and Hall, 1982; Shur, 1988). Glycosidases normally function by binding an appropriate substrate, cleaving the linkage between sugar molecules and subsequently releasing a sugar molecule. In order for a glycosidase to function as a receptor in sperm-egg recognition, it would be necessary that the catalytic cycle of the enzyme to be unable to complete itself proceeding only to the point where the glycosidase has formed a complex with the sugar molecule but has not cleaved it from the glycan.

Many organisms have glycosidases present on the the sperm plasma membrane including mannosidases in mammals, fucosidases and N-acetylglucosaminidases in tunicates, and mannosidases, fucosidases and N-acetylglucosaminidases in flies (Tulsiani et al., 1997; Godknecht and Honegger, 1991; Matsumoto et al., 2002; Downey and Lambert, 1994; Perotti, 2001; Pasini et al., 1999; Intra et al., 2006; Intra et al., 2011; Cattaneo et al., 1997).

In mammals, pretreating sperm with either D-mannose or mannosidase antibodies inhibits sperm-egg binding in a dose dependent manner (Cornwall et al., 1991; Tulsiani, 2000; Tulsiani et al., 1989; Yoshida-Komiya, et al., 1999; Pereira et al., 1998).

In *Ciona intestinalis*, a sperm fucosidase and egg fucose are thought to be involved in sperm-egg binding (Rosati, 1985) because sperm-egg binding is inhibited by the presence of 50 mM L-fucose, pNP-**α**-L-fucose or pNP- **β**-L-fucose (Rosati and De Santis, 1980; Hoshi et al., 1983). Binding competition assays with NAG-glycosides in *Ascidia nigra* and *Phallusia mamillata* led to the conclusion that sperm N-acetylglucosaminidase and an egg N-acetylglucosamine (NAG) are likely involved in sperm-egg recognition (Rosati, 1985; Rosati and de Santis, 1978). There is also evidence that N-acetylglucosaminidase plays a role in *Phallusia mamillata* sperm-egg recognition events. Strangely, knockouts in systems such as UDP-Gal:β-D-Gal-α1,3-Gal-galactosyltransferase interactions in mice are fertile even though they do not produce oocytes with Gal-α1,3-Gal epitopes (Thall et al., 1997). A knockout system that prevents N-acetylglucosamine and galactose addition to eggs did not affect fertilization (Williams et al., 2007). As fertilization is paramount to species fitness, it is likely that a fail-safe mechanism is involved in molecular mechanisms involved in fertilization. One potential fail-safe mechanism is a built in redundancy in the system.

Less is known about glycosidase involvement in insect sperm-egg recognition events. In fact, how gametes interact with each other has only recently been explored in genera within Diptera (Drosophilidae and Tephritidae) and there is no conclusive answer as to the molecular mechanism of sperm-egg interactions in insect species. *Drosophila*

sperm possess α-L-fucosidase, α-mannosidase and β-N-acetylhexosaminidase and the egg micropyle is coated with the cognate sugars suggesting a possible role in the sperm-egg interactions (Intra et al., 2006; Intra et al., 2011; Perotti, 2001). A *Casanova* mutant lacking β-N-acetylhexosaminidase on the sperm head is unable to penetrate the egg micropyle (Perotti, 2001). Sperm from the Mediterranean fruit fly, *Ceratitis capitata,* also have glycosidases on the plasma membrane and the respective glycoconjugates on the egg surface (Intra et al., 2011).

Questions to be addressed

This dissertation addresses questions related to the movement of sperm and other proteins through the male reproductive tracts of insects including sperm-egg recognition and motility regulation in water striders and mosquitoes, respectively.

1. How is sperm movement from the testes to the seminal vesicles regulated? Hypothesis: *npyr*, *ssr*, and *5-htr* are all transcribed in the testes of *Culex* mosquitoes. These three receptors have been implicated in regulating circadian rhythms in mammals. Also, moths have been shown to use circadian rhythms to regulate sperm movement out of the testes. Therefore, I hypothesize that circadian rhythms are involved in moving sperm from the testes to the seminal vesicles.
2. How is sperm movement from the seminal vesicles to the female regulated? Hypothesis: In mammals, the male reproductive tract requires muscle contractions in the vas deferens and seminal vesicles. In insects, the female reproductive tract undergoes muscle contractions to move the egg through the tract. Based on this, I

hypothesize that muscle contractions aid in movement of sperm and accessory gland components from the male to female reproductive tract.

3. What are the characteristics of the accessory gland components that are responsible for sperm activation?

   Hypothesis: In *Culex quinquefasciatus* and *Aquarius remigis*, a trypsin like molecule was discovered to initiate motility in vitro and *Culex* accessory gland components can fully stimulate sperm motility (Miyata et al., 2012; Thaler et al., 2014). Based on this, I hypothesize that trypsin-like proteins will be present in the accessory gland extracts and the accessory gland extracts will have trypsin-like activity.

4. What are the characteristics of glycosidases potentially involved in sperm-egg recognition?

   Hypothesis: In other systems, including *Drosophila* and *Ceratitis*, there is evidence that a non-enzymatic interaction between glycosidases and glycoconjugates is involved in sperm-egg interaction. Based on this, I hypothesize that there will be glycosidases present on the *Aquarias remigis* acrosomal plasma membrane that can interact efficiently with glycoconjugates.

The following chapters will detail my investigations into circadian rhythms in the testes, muscle contractions in the accessory glands and seminal vesicles, sperm motility activation, and sperm-egg interactions in the female.

References

Acott T.S. and Carr D.W. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. *Biol. Reprod.* 30: 926-35.

Afzelius B. A. (1959). Electron microscopy of the sperm tail. Results obtained with a new fixative. *J. Biophys. Biochem. Cytol.* 5: 269–278.

Aitken J. and Fisher H. (2005). Reactive oxygen species generation and human spermatozoa: The balance of benefit and risk. *Bioessays.* 16: 259-67.

Alavi S. M. H. and Cosson, J. (2005). Sperm motility in fishes. I. Effects of temperature and pH: a review. *Cell Biol. International* 29: 101–110.

Albrecht U, Sun Z.S., Eichele G and Lee C.C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. *Cell*. 91:1055–1064.

Alvarez J.D., Chen D., Storer E. and Sehgal A. (2003). Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. *Biol. Reprod.* 69: 81–91.

AI-Zuhair A.G.H., Dixon J.S. and Gosling J.A. (1976). Studies on the autonomie innervation of the guinea pig seminal vesicle and ductus deferens. *J. Physiol., Lond.* 257:9P-UP.

Arnqvist G. (1997). The Evolution of Water Strider Mating Systems:Causes and Consequences of Sexual Conflicts. In J. Choe, and B. Crespi, *The Evolution of Mating Systems in Insects and Arachnids* (pp. 146-163). Cambridge: Cambridge University Press.

Asidi, A., N'Guessan R., Curtis C. and Rowland M. (2012). Loss of household protection from use of insecticide-treated netsagainst pyrethroid-resistant mosquitoes, *Benin. Emerg. Infect. Dis.*18: 1101–1106.

Avila F.W., Wolfner M,F. (2009). Acp36DE is required for uterine conformational changes in mated Drosophila females. *Proc. Natl. Acad. Sci. USA.* 106:15796–800

Baba T., Azuma S., Kashiwabara S., Toyoda Y. (1994a). Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *J. Biol. Chem.* 269: 31845-31849.

Baba, T., Niida, Y., Michikawa, Y., Kashiwabara, S., Kodaira, K., Takenaka, M., Kohno, N., Gerton, G.L., Arai, Y. (1994b). An acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two proacrosins and an acrosin intermediate. *J. Biol. Chem.* 269: 10133-10140.

Baccetti B. (1972). Insect sperm cells. *Adv. Insect Phys.* 9: 315–397.
Bacetti and Afzelius. (1976). The biology of the sperm cell. S. Karger, Besel.
Bacetti (1979). Ultrastructure of sperm and its bearing on arthropod phylogeny. Pages 609-44 In A. P. Gupta, Ed. Arthropod phylogeny. Van Nostrand Reinhold Co., New York.

Baccetti B., Gibbons, B.H. and Gibbons, I. R. (1989). Bidirectional swimming in spermatozoa of tephritid flies. *J. Submicrosc. Cytol. Pathol.* 21: 619–626.

Bacq Z.M. (1931). Impotence of the male rodent after sympathetic denervation of the genital organs. *Am. J. Physiol.* 96: 321-330.

Baker M., Witherdin R., Hetherington, L., Cunningham-Smith, K., and Aitken, R. (2005). Identification of post-translational modifications that occur during sperm maturation using difference in two-dimensional gel electrophoresis. *Proteomics. 5*: 1003-1012.

Balsalobre A., Brown S.A., Marcacci L., Tronche F., Kellendonk C., Reichardt H.M., Schutz G. and Schibler U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*. 289: 2344 – 2347.

Baltz J. and Cardullo R. (1989). On the number and rate of formation of sperm–zona bonds in the mouse. *Gamete Research. 24*: 1-8.

Bamji S.X., Orchard I. (1995). Pharmacological profile of octopamine and 5HT receptor on the lateral oviducts of the cockroach, *Periplaneta americana*. *Arch. Ins. Bioch. Phys.* 28: 49–62.

Beaver L.M., Gvakharia B.O., Vollintine T.S., Hege D.M., Stanewsky R. and Giebultowicz J.M. (2002). Loss of circadian clock function decreases reproductive fitness in males of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* 299: 2134–2139.

Benoff S., Cooper G.W., Hurley I., Napolitano B., Rosenfeld D.L., Scholl G.M. and Hershlag A. (1993a). Human sperm fertilizing potential in vitro is correlated with differential expression of a head-specific mannose-ligand receptor. *Fertility and sterility.* 59: 854-862.

Benoff S., Hurley I., Cooper G.W., Mandel F.S., Hershlag A., Scholl G.M. and Rosenfeld D.L. (1993b). Fertilization potential in vitro is correlated with head-specific mannose-ligand receptor expression, acrosome status and membrane cholesterol content. *Human reproduction* (Oxford, England) 8: 2155-2166.

Bentley J.K., Khatra A.S. and Garbers D.L. (1988). Receptor-mediated activation of detergent-soluilized guanylate cyclase. *Biol. Reprod*. 39: 639-47.

Birowo P., Uckert S., Kedia G.T. and Sonnenberg J.E. (2010). Characterization of the effects of various drugs likely to affect smooth muscle tension on isolated human seminal vesicle tissue. *Urology*. 75:974- 978.

Birowo P., Uckert S., Kedia G.T. and Sonnenberg J.E. (2010). Exposure of human seminal vesicle tissue to phosphodiesterase (PDE) inhibitors antagonizes the contraction induced by norepinephrine and increases production of cyclic nucleotides. *Urology*. 76: 1518.e1-1518.e6.

Bishop G. H. (1920). Fertilization in the honeybee. II. Disposal of the sexual fluids in the organs of the female. *J. Exp. Zool*. 31: 267-86.

Bittman E.L., Doherty L.S., Huang L. and Paroskie A. (2003). *Period* gene expression in mouse endocrine tissues. *Am. J. Physiol.* 285: R561–R569.

Bleil J.D. and Wassarman P.M., (1983). Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* 95: 317-324.

Bleil J.D. and Wassarman P.M., (1990). Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. *Proc. Natl. Acad. Sci. USA.* 87: 5563-5567.

Bloch Qaz, M. C., Aprille J. R. and Lewis S. M. (1998). Female role in sperm storage in the red flour beetle, *Tribolium castaneum. Comp. Biochem. Physiol.* A120: 641-647.

Bloch Qazi M. C., Heifetz Y. and Wolfner M. F. (2003). The developments between gametogenesis and fertilization: ovulation and female sperm storage in *Drosophila melanogaster. Dev. Biol.* 256: 195-211.

Boerke A., Dieleman S.J. and Gadella B.M. (2007). A possible role for sperm RNA in early embryo development. *Theriogenology.* 68: S147–S155.

Bressac C., Joly D., Devaux J., Serres C., Feneux D. and Lachaise D. (1991). Comparative kinetics of short and long sperm in sperm dimorphic *Drosophila species*. *Cell Motil Cytoskeleton.* 19: 269–274.

Campbell V., and Fairbairn D. (2001). Prolonged copulation and the internal dynamics of sperm transfer in the water strider *Aquarius remigis*. *Can. J. Zool. 79*: 1801–1812.

Cardullo R.A. and Wolf D.E. (1991). The Sperm Plasma Membrane. A little More than Mosaic, a Little Less than Fluid. In: RA Bloodgood, ed Ciliary and Flagellar Membranes Plenum Press, New York. 305-336.

Carr D.W., Usselman M.C. and Acott T.S. (1985): Effects of pH, lactate, and viscoelastic drag on sperm motility: a species comparison. *Biol. Reprod*. 33: 588-95.

Cattaneo F., Pasini M., and Perotti M. (1997). Glycosidases are present on the surface of *Drosophila melanogaster* spermatozoa. . *Mol. Reprod. Dev. 48* (2): 276-281.

Chiu P.C., Chung M.K., Koistinen R., Koistinen H., Seppala M., Ho P.C., Ng E.H., Lee K.F. and Yeung W.S. (2007). Glycodelin-A interacts with fucosyltransferase on human sperm plasma membrane to inhibit spermatozoa-zona pellucida binding. *J. Cell Sci.* 120: 33-44.

Chen Y., Cann M.J., Litvin T.N., Iourgenko V., Sinclair M.L., Levin L.R. and Buck J. (2000). Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science.* 289: 625-8.

Cheng A., Le T., Palacios M., Bookbinder L.H., Wassarman P.M., Suzuki F. and Bleil J.D. (1994). Sperm-egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. *J. Cell Biol.* 125: 867-878.

Christen R., Schackmann R.W. and Shapiro B.M. (1982): Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin, *Strongylocentrotus purpuratus. J. Biol. Chem.* 257, 14881-90.

Clark J and Lange A.B. (2003). Octopamine modulates spermathecal muscle contractions in *Locusta migratoria*. *J. Comp. Physiol. A. Neuroethol. Sens. Neural Behav. Physiol.* 189:105-114.

Cook B.J., and Meola S. (1978). The oviduct musculature of the horsefly, *Tabunus sulcfrons*, and its response to 5-hydroxytryptamine and proctolin. *Physiol. Entomol.* 3:273-280.

Cook B.J. and Wagner R.M. (1992). Some pharmacological properties of the oviduct muscularis of the stable fly, *Stomoxys calcitrans. Comp. Biochem. Physiol. C.* 102: 273-280.

Cottrell, Amadesi, Schmidlin and Bunnett. (2003). Protease-activated receptor 2: activation, signalling and function. *Biochemical Society Transactions* 31, 6

Corbel V., N'Guessan R., Brengues C., Chandre F., Djogbenou L., Martin T., Akogbeto M., Hougard J.M. and Rowland M. (2007). Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica*. 101: 207–216.

Cornwall G., Tulsiani D., and Orgebin-Crist M. (1991). Inhibition of the mouse sperm surface alpha-D-mannosidase inhibits sperm-egg binding in vitro. *Biol. Reprod. 44* (5): 913-921.

Curtis S. K. and Benner D. B. (1991). Movement of spermatozoa of *Megaselia scalaris* (Diptera: Brachycera: Cyclorrhapha: Phoridae) in artificial and natural fluids. *J. Morphol.* 210: 85–99.

Damiola F., Le Minh N., Preitner N., Kornmann B., Fleury-Olela F. and Schibler U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* 14:2950–2961.

Dangott L.J. and Garbers D.L. (1984). Identification and partial characterization of the receptor for speract. *J. Biol. Chem*. 22: 13712-6.

Darszon A., Labarca P., Nishigaki T. and Espinosa F. (1999). Ion channels in sperm physiology. *Physiol. Rev.* 79: 481-510.

Davey K. G. (1965). Reproduction in the Insects. San Francisco: Freeman. 96 pp.
Davis N. T. (1965). Studies of the reproductive physiology of Cimicidae. II. Artificial insemination and the function of the seminal fluid. *J. Insect Physiol*. 11: 355-66.

Degrugillier M. E. and Leopold R. A. (1976). Ultrastructure of sperm penetration of house fly eggs. *J. Ultrastruct. Res*. 56: 312–325.

Downey J., and Lambert C. (1994). Attachment of the ascidian sperm surface egg receptor N- acetylglucosaminidase to the cell membrane. *Mol. Reprod. Dev. 38* (4): 453-458.

Dunbar B. S., Avery S., Lee V., Prasad S., Schwahn D., Schwoebel E., Skinner S., and Wilkins B. (1994). The mammalian zona pellucida: Its biochemistry, immunochemistry, molecular biology, and developmental expression. *Reprod. Fertil. Dev.* 6: 331–347.

Eliasson R. and Risley P.L. (1966). Potentiated response of isolated seminal vesicles to catecholamines and acetylcholine in the presence of PGE. *Ada. Physiol. Scand.* 67: 253-254.

Ensslin M.A. and Shur B.D., (2003). Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin-domain protein involved in sperm-egg binding. *Cell.* 114: 405-417.

Falck B., Owman C. and Sjöstrand, N.O. (1965). Peripherally located adrenergic neurons innervating the vas deferens and the seminal vesicle of the guinea-pig. *Experientia* 21: 98-100.

Florman H.M. and Wassarman P.M. (1985). O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. *Cell* 41: 313-324.

Focarelli R., La Sala G., Balasini M., and Rosati F. (2001). Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatus* model. *Cells Tissues Organs. 168*: 76-81.

Foster L.J., De Hoog C.L., Mann M. (2003). Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. USA*. 100: 5813-5818.

Franzén A., (1956). On spermiogenesis, morphology of the spermatozoon and biology of fertilization among invertebrates. *Zool. Bidr. Uppsala.* 31: 355-482.

Gaddum P. (1968). Sperm maturation in the male reproductive tract: Development of motility. *The Anatomical Record, 161* (4): 471-482.

Gadella B.M., Lopes-Cardozo M., van Golde L.M., Colenbrander B. and Gadella Jr. T.W. (1995). Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction. Evidence for a primary capacitation event in boar spermatozoa. *J. Cell Sci*. 108: 935–946.

Gibbons I. R. and Fronk E. (1972). Some properties of bound and soluble dynein from sea urchin sperm flagella. *J. Cell Biol.* 54: 365–381.

Gibson. (1985). Swarming behaviour of the mosquito *Culex pipiens quinquefasciatus*: a quantitative analysis. *Physiol. Entom.*10: 283-296.

Giebultowicz, Bell and Imberski. (1988). Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol.* 34(6): 521-532.

Giebultowicz J.M. and Riemann J.G. (1990). Circadian system in the insect testes controls the rhythmic release of sperm. *Prog. Clin. Biol. Res.* 341B: 655–660.

Giebultowicz J.M. and Joy J.E. (1992). Ontogeny of the circadian system controlling release of sperm from the insect testis. *J. Biol. Rhythms* 7: 203–212.

Gillott C. (1988). Arthropoda-Insecta. In Reproductive Biology of the Invertebrates, vol, III, Accessory Sex Glands (ed. K. G. Adiyodi and R. G. Adiyodi), pp. 319-471. New York: John Wiley and Sons

Glabe C., and Vacquier V. (1978). Egg surface glycoprotein receptor for sea urchin sperm bindin. *Proc. Natl. Acad. Sci. USA, 75* (2): 881-885.

Godknecht A., and Honegger T. (1991). Isolation, Characterization, and Localization of a Sperm-Bound N-Acetylglucosaminidase that Is Indispensable for Fertilization in the Ascidian, *Phallusia mammillata*. *Dev. Biol. 143*: 398-407.

Goldman B.D. (1999). The circadian timing system and reproduction in mammals. *Steroids*. 64: 679–685.

Giuliano F. (2007). 5-Hydroxytryptamine in premature ejaculation: opportunities for therapeutic intervention. *Trends Neurosci*. 30: 79–84.

Gvakharia B.O., Kilgore J.A., Bebas P. and Giebultowicz J.M. (2000). Temporal and spatial expression of the period gene in the reproductive system of the codling moth. *J. Biol. Rhythms.* 15: 4–12.

Habermann F., and Sinowatz F. (2011). Glycobiology of Fertilization and Early Embryonic Development. In H. Gabius, *The Sugar Code: Fundamentals of Glycosciences.* Weinheim: Wiley-VCH Verlag GmbH and Co KGaA, Weinheim.

Hardy C.M., Clydesdale G. and Mobbs, K.J. (2004). Development of mouse-specific contraceptive vaccines: infertility in mice immunized with peptide and polyepitope antigens. *Reproduction.* 128: 395-407.

Hess B., Saftig P., Hartmann, D., Coenen R., Lullmann-Rauch R., Goebel H.H., Evers M., von Figura K., D'Hooge R., Nagels, G., De Deyn, P., Peters, C. and Gieselmann, V. (1996). Phenotype of arylsulfatase A-deficient mice: relationship to human metachromatic leukodystrophy. *Proc. Natl. Acad. Sci. USA*. 93: 14821-14826.

Hib, Ponzio and Vilar. (1984). Effects of autonomic drugs on contractions of rat seminal vesicles *in vivo*. *J. Reprod. Fert*. 70:197-202.

Higginson D., Miller K., Segraves K. and Pitnick S. (2012). Female reproductive tract form drives the evolution of complex sperm morphology. *Proc. Natl. Acad. Sci. USA, 109* (12): 4538–4543.

Hoshi M., De Santis R., Pinto M., Cotelli F. and Rosati F. (1983). Is sperm a-L-fucosidase responsible for sperm-egg binding in *Ciona intestinalis*. *The Sperm Cell*. 107-110.

Hsieh JT, Liu SP, Chang HC, Kuo YC, Chen JH, Jaw FS and Tsai VFS. (2010). Parasympathetic influence plays an independent and significant role in inducing the contraction of the seminal vesicle of the rat. *Urology.* 76: 511.e1-511.e4.

Hughes M. and Davey K. G. (1969). The activity of spermatozoa of *Periplaneta. Insect Physiol.* 15: 1607- 16.

Hunnicutt G.R., Mahan K., Lathrop W.F., Ramarao C.S., Myles D.G. and Primakoff P. (1996a). Structural relationship of sperm soluble hyaluronidase to the sperm membrane protein PH-20. *Biol. Reprod.* 54: 1343-1349.

Hunnicutt G.R., Primakoff P. and Myles, D.G., (1996b). Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. *Biol. Reprod.* 55: 80-86.

Ikawa M., Nakanishi T., Yamada S., Wada I., Kominami K., Tanaka H., Nozaki M., Nishimune Y. and Okabe M. (2001). Calmegin is required for fertilin alpha/beta heterodimerization and sperm fertility. *Dev. Biol.* 240: 254-261.

Inoue M. and Wolf, D.P. (1975). Fertilization-associated changes in the murine zona pellucida: a time sequence study. *Biol. Reprod.* 13: 546-551.

Intra J., Caro D., Perotti M. and Pasini, M. (2011). Glycosidases in the plasma membrane of *Ceratitis capitata* spermatozoa. *Insect Biochem. Mol. Biol*. *41*: 90-100.

Intra J., Cenni F. and Perotti, M. (2006). An a-L-Fucosidase Potentially Involved in Fertilization Is Present on *Drosophila* Spermatozoa Surface. *Mol. Reprod. Dev. 73*: 1149-1158.

Ito S. (1966). Movement and structure of louse spermatozoa. *J. Cell Biol.* 31: 128A.

Jamieson B. (1987). *The Ultrastructure and Phylogeny of Insect Spermatozoa.* New York, NY: Cambridge University Press.

Jamieson B.G.M., Dallai R. and Afzelius B.A. (1999). Insects: Their Spermatozoa and Phylogeny. USA: Science Publishers. Pp 1–23.

Johnson C.H., Clapper D.L., Winkler M.M., Lee H.C. and Epel D. (1983). A volatile inhibitor immobilizes sea urchin sperm in semen by depressing the intracellular pH. *Dev. Biol.* 98: 493–501.

Jones R. (1989). Membrane remodelling during sperm maturation in the epididymis. Oxf Rev Reprod Biol. 11, 285-337.

Jones A.K. and Sattelle D.B. 2010. Diversity of Nicotinic Acetylcholine Receptor Subunits. In: Insect Nicotinic Acetylcholine Receptors, Volume 683 ,(ed. Thany SH), Pp. 25-43. Texas, USA. Landes Bioscience

Kamei N., and Glabe C. (2003). The species-specific egg receptor for sea urchin sperm adhesion is EBR1,a novel ADAMTS protein. *Genes Dev. 17* (20): 2502-2507.

Karr J.P. and Almquist J.O. (1973) A new technique for *in vivo* measurement of accessory sex organ activity in male rabbits. *J. Anim. Sci.* 36: 554-562.

Kennaway D.J. (2005). The role of circadian rhythmicity in reproduction. *Human Reprod. Update.* 11(1): 91–101.

Khan N. R. and Musgrave A. J. (1969). Observations on the functional anatomy of the reproductive organs of Sitophilus. *Can. J. Zool*. 47:665-69

Kim K.S., Cha M.C. and Gerton G.L., (2001). Mouse sperm protein sp56 is a component of the acrosomal matrix. *Biol. Reprod.* 64: 36-43.

Kimura T., Yasuyama K. and Yamguchi T. (1989). Proctolinergic innervation of the accessory gland in male crickets (*Gryllus bimaculatus*): detection of proctolin and some pharmacological properties of myogenically and neurogenically evoked contractions. *J. Insect Physiol.* 35: 251–264.

King D.P., Zhao Y., Sangoram A.M., Wilsbacher L.D., Tanaka M., Antoch M.P., Steeves T.D., Vitaterna M.H., Kornhauser J.M., Lowrey P.L., Turek F.W. and Takahashi J.S. (1997). Positional cloning of the mouse circadian clock gene. *Cell.* 89:641–653.

Kobayashi K., Kanno S., Smit B., van der Horst G.T., Takao M. and Yasui A. (1998). Characterization of photolyase/blue-light receptor homologs in mouse and human cells. *Nucleic Acids Res.* 26: 5086–5092.

Koeniger G. (1970). Die Bedeutung der Tracheenhu¨ lle und der Anhangsdru¨se der Spermatheka fu¨ r die Befruchtungsfa¨higkeit der Spermatozoen in der Bienenko¨nigin. *Apidologie* 1: 55–71.

Köttgen M., Hofherr A., Li W., Chu K., Cook S., Montell C. and Watnick T. 2011. Drosophila sperm swim backwards in the female reproductive tract and are activated via TRPP2 ion channels. PLoS One 6(5): e20031.

LaChance L. E., Richard R. D. and Ruud R. L. (1977) Movement of eupyrene sperm bundles from the testes and storage in the ductus ejaculatoris duplex of the male pink bollworm: Effects of age, strain, irradiation and light. *Ann. Ent. Soc. Am*. 70, 647-651.

Landa V. (1960). Origin, development and function of the spermatophore in cockchafer *(Melolontha melolontha L.). Acta Soc. Entomol. Gech.* 57: 297-316.

Landa V. (1961). Experiments with an artificial spermatophore in cockchafer *(Melolontha melolontha L.). Acta Soc. Entomol. tech.* 58(4): 297-301.

Lange A.B. and Tsang P.K.C. (1993). Biochemical and physiological effects of octopamine and selected octopamine agonists on the oviducts of *Locusta migratoria*. *J. Insect Physiol.* 39: 393-400.

Lange. (2003). A Neurohormonal Role for Serotonin in the Control of Locust Oviducts. Archives of Insect Biochemistry and Physiology 56:179–190

Lee H.G., Seong C.S., Kim Y.C., Davis R.L. and Han K.A. (2003) Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. Dev Biol. 264:179-190.

Leopold R. A. and Degrugillier M.E. (1973). Sperm penetration of house fly eggs: evidence for involvement of a female accessory secretion. *Science.* 181: 555 - 57.

Levine N. and Kelly H. (1978): Measurement of pH in the rat epididymis *in vivo*. J. *Reprod. Fertil.* 52: 333-5.

Li T. and Liu N. (2010). Inheritance of permethrin resistance in *Culex quinquefasciatus. J. Med. Entomol.* 47: 1127–1134.

Linley J.R. (1979). Activity and motility of spermatozoa of *Culicoides melleus* (Diptera: Ceratopogonidae). *Entomologia Experimentalis et Applicata* 26: 85–96.

Lishko P.V., Botchkina I.L., Fedorenko A. and Kirichok Y. (2010). Acid extrusion from human spermatozoa is mediated by flagellar voltage-gated proton channel. *Cell*. 140: 327-37.

Litscher E.S., Juntunen K., Seppo A., Penttila L., Niemela R., Renkonen O. and Wassarman P.M., (1995). Oligosaccharide constructs with defined structures that inhibit binding of mouse sperm to unfertilized eggs in vitro. *Biochemistry* 34: 4662-4669.

Lopez, L., and Shur, B. (1987). Redistribution of mouse sperm surface galactosyltransferase after the acrosome reaction. *J. Cell Biol., 105*: 1663-1670.

Lopez L., Bayna E., Litoff D., Shaper N., Shaper J. and Shur B. (1985). Receptor function of mouse sperm surface galactosyltransferase during fertilization. *J. Cell Biol., 101*: 1501-1510.

Matsumoto M., Hirata J., Hirohashi N., and M H. (2002). Sperm-Egg Binding Mediated by Sperm Alpha-L-Fucosidase in The Ascidian *Halocynthia roretzi*. *Zool. Science, 19*: 43-48.

McGraw E.A. and O'Neill S.L. (2013). Beyond insecticides: new thinking on an ancient problem. *Nat. Rev. Microbiol*. 11: 181–193

Mencarelli C., Lupetti, Rosetto and Dallai. (2000). Morphogenesis of the giant sperm axoneme in *Asphondylia ruebsaameni* Kertesz (Diptera, Cecidomyiidae). *Tissue and Cell*. 32 (2): 188–197.

Mengerink K., and Vacquier V. (2001). Glycobiology of Sperm-Egg Interactions in Deuterostomes. *Glycobiology , 11* (4): 37-43.

Middleton C.A., Nongthomba U., Parry K., Sweeney S., Sparrow J. and Elliott C. (2006). Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. BMC Biol. 4, 17

Miller G. and Pitnick S. (2002). Sperm-Female Coevolution in Drosophila. *Science, 298*: 1230-1233.

Miyata H., Noda N., Fairbairn D., Oldenbourg R. and Cardullo R. (2011). Assembly of the Fluorescent Acrosomal Matrix and its Fate in Fertilization in the Water Strider, *Aquarius remigis*. *Journal of Cell Physiology , 226* (4), 999-1006.

Miyata H., Thaler C., Haimo L. Cardullo R. (2012). Protease Activation and the Signal Transduction Pathway Regulating Motility in Sperm from the Water Strider *Aquarius remigis. Cytoskeleton*. 69: 207–220

Monastirioti M. (2003) Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila melanogaster. Dev Biol 264:38-49.

Morse D., Cermakian N., Brancorsini S., Parvinen M. and Sassone-Corsi P. (2003). No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. *Mol. Endocrinol*. 17:141–151.

Naz R.K., Brazil C. and Overstreet, J.W. (1992). Effects of antibodies to sperm surface fertilization antigen-1 on human sperm-zona pellucida interaction. *Fertility and sterility* 57: 1304-1310.

Naz R.K., Rosenblum B.B. and Menge A.C. (1984). Characterization of a membrane antigen from rabbit testis and sperm isolated by using monoclonal antibodies and effect of its antiserum on fertility. *Proc. Natl. Acad. Sci. USA*. 81: 857-861.

Neill A.T. and Vacquier V.D. (2004): Ligands and receptors mediating signal transduction in sea urchin spermatozoa. *Reproduction.* 127: 141-9.

Neubaum D. M. and Wolfner M. F. (1999). Wise, winsome or weird: mechanisms of sperm storage in female insects. *Curr. Top. Dev. Biol*. 41: 67-97.

Nojimoto F.D., Piffer R.C., Kiguti L.R., Lameu C., de Camargo A.C., Pereira O.C. and Pupo A.S. (2009). Multiple effects of sibutramine on ejaculation and on vas deferens and seminal vesicle contractility. *Toxicol. Appl. Pharmacol*. **239**: 233–240.

Nykamp D.A. and Lange A.B. (2000). Interaction between octopamine and proctolin on the oviducts of *Locusta migratoria*. *J. Insect Physiol*. 46:809-816.

Omura S. (1936). Artificial insemination of *Bombyx mori. J. Fac. Agric. Hokkaido Imp. Univ. Sapporo.* 38: 135-50.

Omura S. (1938). Studies On the reproductive system of the male *Bombyx mori.* II. Post-testicular organs and post-testicular behavior of the spermatozoa. *J. Fac. Agric. Hokkaido Imp. Univ. Sapporo* 40: 129-70.

Osanai M. and Baccetti B. (1993). Two-step acquisition of motility by insect spermatozoa. *Experientia (Basel)* 49: 593–595.

Ott G.J., Shirkey N.J., Haimo L.T. Cardullo R.A. and Thaler C.D. (2015). Germ-cell hub position in a heteropteran testis correlates with the sequence and location of spermatogenesis and production of elaborate sperm bundles. *Mol. Reprod. Dev. 82(4):* 295–304

Parks J.E. and Hammerstedt R.H. (1985). Development changes occurring in the lipids of ram epididymal spermatozoa plasma membrane. *Biol. Reprod* 32: 653–68.

Pasini M., Cattaneo F., Pinto M., De Santis R. and Perotti, M. (1999). Plasma membrane association and preliminary characterization of *Drosophila* sperm surface glycosidases. *Mol. Reprod. Dev. 52*: 166-173.

Pereira B., Abou-Haila A. and Tulsiani D. (1998). Rat Sperm Surface Mannosidase Is First Expressed on the Plasma Membrane of Testicular Germ Cells. *Biol. Reprod. 59*: 1288–1295.

Perotti M. C. (2001). Male sterile mutant casanova gives clues to mechanisms of sperm-egg interactions in *Drosophila melanogaster*. *Mol. Reprod. Dev. 60* (2): 248-259.

Phelps B.M., Primakoff P., Koppel D.E., Low M.G., Myles D.G., (1988). Restricted lateral diffusion of PH-20, a PI-anchored sperm membrane protein. *Science.* 240: 1780–1782.

Phillips D. M. (1974). Structural variants in invertebrate sperm flagella and their relationship to motility. In Cilia and Flagella (ed. M. A. Sleigh), pp. 379–402. Academic Press, London, New York.

Pitnick S., Spicer G. S. and Markow T. A. (1995). How long is a giant sperm? *Nature.* 375: 109.

Pratt S. A. (1968). An electron microscope study of Nebenkern formation and differentiation in spermatids of *Murgantia histrionica* (Hemiptera, Pentatomidae). *J. Morphol.* 126: 31–66.

Presgraves D., Baker R., and Wilkinson G. (1999). Coevolution of sperm and female reproductive tract morphology in stalk-eyedies. *The Royal Society*. 1041-1047.

Quicke D.L.J., Ingram S.N., Baillie H. S. and Gaitens P. V. (1992). Sperm structure and ultrastructure in the Hymenoptera (Insecta). *Zoologica Scripta* 21: 381–402.

Ramachandran R., Mihara K., Mathur M., Rochdi M.D., Bouvier M., Defea K., Hollenberg M.D. (2009). Agonist-biased signaling via proteinase activated receptor-2: differential activation of calcium and mitogen-activated protein kinase pathways. *Mol. Pharmacol*. 76:791–801.

Rankin S.M., TeBrugge V.A., Murray J.A., Schuler A.M. and Tobe S.S. (2009). Effects of selected neuropeptides, mating status and castration on male reproductive tract movements and immunolocalization of neuropeptides in earwigs. *Comp. Biochem. Phys. A.* 152: 83 -90

Ranson H., N'guessan R., Lines J., Moiroux N., Nkuni Z. and Corbel V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol.* 27: 91–98.

Rao H.V. and Davis N.T. (1969). Sperm activation and migration in bed bugs. *J. Insect Physiol.* 15: 1815-32.

Ribou and Reinhardt. (2012). Reduced metabolic rate and oxygen radicals production in stored insect sperm. *Proc. R. Soc. B.* 279.

Rosano G., Caille A.M., Gallardo-Rios M. and Munuce M.J., (2007). D-Mannose-binding sites are putative sperm determinants of human oocyte recognition and fertilization. *Reproductive biomedicine online* 15: 182-190.

Rosati F. (1985). Sperm egginteraction in ascidians. In Metz, and Monroy, *Biology of fertilization.* (Vol. 2, p. 361 384). New York: Academic Press.

Rosati F., and De Santis R. (1978). Studies on fertilization in the Ascidans. I. Self-sterility and specific recognition between gametes of *Ciona intestinalis*. *Exp. Cell Res., 112* (1): 111-119.

Rosati F., and De Santis R. (1980). Role of the surface carbohydrates in sperm-egg interaction in *Ciona intestinalis*. *Nature. 283* (5749): 762-764.

Rubenstein D. (1989). Sperm competition in water strider, *Gerris remigis*. *Animal Behavior. 38*: 631-636.

Sacco A.G., Yurewicz E.C., Subramanian M.G. and Matzat P.D., (1989). Porcine zona pellucida: association of sperm receptor activity with the alpha-glycoprotein component of the Mr = 55,000 family. *Biol. Reprod.* 41: 523-532.

Sangoram A.M., Saez L., Antoch M.P., Gekakis N., Staknis D., Whiteley A., Fruechte E.M., Vitaterna M.H., Shimomura K., King D.P., Young M.W., Weitz C.J. and Takahashi J.S. (1998). Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCKBMAL1- induced transcription. *Neuron.* 21:1101–1113.

Satir P. (1965). Studies on cilia. II. Examination of the distal region of the ciliary shaft and the role of the filaments in motility. *J. Cell Biol.* 26: 805–834.

Satir P. (1968). Studies on cilia. III. Further studies on the cilium tip and a ‘‘sliding filament’’ model of ciliary motility. *J. Cell Biol.* 39: 77–94.

Sharma P., Varma R., Sarasij R.C., Ira Gousset K., Krishnamoorthy G., Rao M. and Mayor S., (2004). Nanoscale organization of multiple GPI-anchored proteins in living cell membranes. *Cell.* 116: 77–89.

Shearman L.P., Zylka M.J., Weaver D.R., Kolakowski L.F. Jr. and Reppert S.M. (1997). Two period homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* 19:1261–1269.

Shepherd J.D. (1974). Activation of saturniid moth sperm by a secretion of the male reproductive tract. *J. Insect Physiol*. 20: 2107-22.

Shur B., and Hall N. (1982). A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J. Cell Biol. 95*: 574-579.

Shur B. (1988). Galactosyltransferase as a recognition molecule during fertilization and development. In H. Schatten, and G. Schatten, *The Molecular Biology of Fertilization.* New York: Academic Press, in press.

Shum W.W.C., Da Silva N., Brown D. and Breton S. (2009): Regulation of luminal acidification in the male reproductive tract via cell-cell crosstalk. *J. Exp. Biol.* 212: 1753-61.

Shur B., Rodeheffer, C., Ensslin M., Lyng R. and Raymond A. (2006). Identification of novel gamete receptors that mediate sperm adhesion to the egg coat . *Mol. Cell Endo. 250*: 137-148.

Sih A. and Watters J. (2005). The mix matters: behavioural types and group dynamics in water striders. *Behaviour*. *142*: 1417-1431.

Sinowatz F., Plendl J. and Kolle S. (1998). Protein-Carbohydrate Interactions During Fertilization. *Acta. Anat. 161*: 196-205.

Sjöstrand N.O. (1965). The adrenergic innervation of the vas deferens and accessory male genital glands. *Ada physiol. scand. Suppl.* 257: 1-82.

Stokkan K.A., Yamazaki S., Tei H., Sakaki Y. and Menaker M. (2001). Entrainment of the circadian clock in the liver by feeding. *Science.* 291:490– 493.

Stoya G. and Penzlin H. (1988). The effect of different putative transmitters on spontaneous phasic contractions of cockroach oviducts. Zool J Physiol 92:301–311.

Stubbs J.D., Lekutis C., Singer K.L., Bui A., Yuzuki D., Srinivasan U. and Parry, G. (1990). cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequences. *Proc. Natl. Acad. Sci. USA*. 87: 8417-8421.

Sun Z.S., Albrecht U., Zhuchenko O., Bailey J., Eichele G. and Lee C.C. (1997). RIGUI, a putative mammalian ortholog of the *Drosophila* period gene. *Cell.* 90:1003–1011.

Swan M. A. (1981). The generation and propagation of double waves in mosquito (*Aedes notoscriptus*) sperm tails. *Gamete Research* 4: 241–250.

Swanson W. J. and Vacquier V. D. (1995b). Liposome fusion induced by a M(r) 18,000 protein localized to the acrosomal region of acrosome-reacted abalone spermatozoa. *Biochemistry* 34: 14202–14208.

Sullivan R., Frenette G. and Girouard, J. (2007). Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit. *Asian J. Androl.* 9: 483–491.

Suzuki-Toyota F., Maekawa M., Cheng A. and Bleil, J.D. (1995). Immuno-colloidal gold labeled surface replica, and its application to detect sp56, the egg recognition and binding protein, on the mouse spermatozoon. *J. Electron Microsc*. 44: 135-139.

Takumi T., Taguchi K., Miyake S., Sakakida Y., Takashima N., Matsubara C., Maebayashi Y., Okumura K., Takekida S., Yamamoto S., Yagita K., Yan L., Young M.W. and Okamura H. (1998). A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. *EMBO J.* 17:4753–4759.

Takumi T., Matsubara C., Shigeyoshi Y., Taguchi K., Yagita K., Maebayashi Y., Sakakida Y., Okumura K., Takashima N. and Okamura H. (1998). A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. *Genes Cells.* 3:167–176.

Tandler B. and Moriber L. (1966). Microtubular structures associated with the acrosome during spermiogenesis in the Water-strider, *Gerris remigis* (Say). *J. Ultrastruct. Res. 14*: 391-404.

Taylor H. C. and Holwill M. E. J. (1999). Axonemal dynein - a natural molecular motor. *Nanotechnology*. 10: 237–243.

Tei H., Okamura H., Shigeyoshi Y., Fukuhara C., Ozawa R., Hirose M. and Sakaki Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. *Nature.* 389:512–516.

Tesarik J., Mendoza C. and Carreras A. (1991). Expression of D-mannose binding sites on human spermatozoa: comparison of fertile donors and infertile patients. *Fertility and sterility.* 56: 113-118.

Thaler C., Miyata H., Haimo L. and Cardullo R. (2014). Waveform Generation Is Controlled by Phosphorylation and Swimming Direction Is Controlled by Ca^{2+} in Sperm from the Mosquito *Culex quinquefasciatus. Biol. Reprod.* 89(6): 1-11.

Thall A., Maly P., and Lowe J. (1995). Oocyte Gal-1,3Gal Epitopes Implicated in Sperm Adhesion to the Zona Pellucida Glycoprotein ZP3 Are Not Required for Fertilization in the Mouse. *J. Biol. Chem. 270* (37): 21437-21440.

Thorson B. J. and Riemann J. G. (1977) Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J. Insect Physiol*. 23: 1189-I 197.

Tian J., Gong H., Thomsen G. and Lennarz, W. (1997). Gamete Interactions in *Xenopus laevis*: Identification of Sperm Binding glycoproteins in the Egg Vitelline Envelope. *J. Cell Biol. 136*: 1099-1108.

Tong Y., Guo H., Brewer J.M., Lee H., Lehman M.N. and Bittman E.L. (2004). Expression of haPer1 and haBmal1 in Syrian hamsters: heterogeneity of transcripts and oscillations in the periphery. *J. Biol. Rhythms.* 19: 113–125.

Tulsiani D.R., Skudlarek M.D. and Orgebin-Crist, M.C. (1989). Novel alpha-D-mannosidase of rat sperm plasma membranes: characterization and potential role in sperm-egg interactions. *J. Cell Biol.* 109: 1257-1267.

Tulsiani D., Yoshida-Komiya H. and Araki Y. (1997). Mammalian Fertilization: A Carbohydrate-Mediated Event. *Biol. Reprod. 57*: 487-494.

Tulsiani D. (2000). Structural Analysis of the Asparagine-Linked Glycan Units of the ZP2 and ZP3 Glycoproteins from Mouse Zona Pellucida. *Arch. Biochem. Biophys. 382* (2): 275-283.

Turek F.W. (1992). Biological rhythms in reproductive processes. *Horm. Res.* 37(suppl 3): 93–98.

Turek F.W., Swann J. and Earnest D.J. (1984). Role of the circadian system in reproductive phenomena. *Recent Prog. Horm. Res.* 40:143–183.

Urch U.A. and Patel H. (1991). The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides. *Development* (Cambridge, England) 111: 1165-1172.

Vacquier V. and Payne J. (1973). Methods for quantitating sea urchin sperm in egg binding. *Exp. Cell Res. 82*: 227-235.

van der Horst G. and Maree L. (2009). SpermBlue: A new universal stain for human and animal sperm which is also amenable to automated sperm morphology analysis. *Biotechnic and Histochemistry*, 1-10.

Van der Spek P.J., Kobayashi K., Bootsma D., Takao M., Eker A.P. and Yasui A. (1996). Cloning, tissue expression, and mapping of a human photolyase homolog with similarity to plant blue-light receptors. *Genomics.* 37:177–182.

Veelaert D., Devreese B., Schoofs L., VanBeeumen J., VandenBroeck J., Tobe S.S., De Loof. (1996). Isolation and characterization of eight myoinhibiting peptides from the desert locust, *Schistocerca gregaria*: new members of the cockroach allatostatin family. *Mol. Cell. Endocrinol.* 122: 183–190.

Villavaso E. J. (1975). The role of the spermathecal gland of the boll weevil, *Anthonomus grandis. J. Insect Physiol.* 21: 1457–1462.

Vinogradova E.B. (2000). *Culex pipiens pipiens* Mosquitoes: Taxonomy, Distribution, Ecology, Physiology, Genetics and Control. Pensoft Publisher, Sofia.

Visconti P.E., Bailey J.L., Moore G.D., Pan D., Olds-Clarke P. and Kopf G.S. (1995): Capacitation of mouse spermatozoa I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development.* 121: 1129-37.

Visconti P.E. and Kopf G.S. (1998): Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod*. 59: 1-6.

Waddel J.A. (1917). The pharmacology of the seminal vesicles. *J. Pharmac. Exp. Ther*. 9: 113-120.

Wassarman P.M. and Litscher E.S. (2008). Mammalian fertilization: the egg's multifunctional zona pellucida. *The International journal of developmental biology* 52: 665-676.

Weigensberg I., and Fairbairn D. (1994). Conflicts of interest between the sexes: a study of mating interactions in a semiaquatic bug. *Animal Behavior*. *48*: 893-901.

Werner M. (2002). Ultrastruktur und Motilita¨t der Spermien von *Aleochara-Arten* (Coleoptera, Staphylinidae). PhD thesis, Albert- Ludwigs-Universita¨t Freiburg i.Br., Germany.

Werner M., Bominghaus A., Zissler D. and Peschke K. (2001). Comparison of sperm structure and motility between *Aleochara tristis* and *A. clavicornis* (Coleoptera, Staphylinidae). *Zoology* 104 (Suppl. IV), 44.

Werner M., Speck T., Zissler D. and Peschke K. (2000). Spermienbewegung beim Kurzflu¨gelka¨fer *Aleochara bilineata* (Coleoptera, Staphylinidae). In Biona-report, vol. 14 (ed. A. Wisser and W. Nachtigall), pp. 45–50. Akademie der Wissenschaften, Mainz.

Werner M. and Simmons L.W. (2008): Insect sperm motility. *Biol. Rev.* 83: 191-208.

WHO. *The World Health Report 2004: Changing History* (WHO, 2004).

Williams S., Xia L., Cummings R., McEver R. and Stanley P. (2007). Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. *J. Cell Science*. *120*: 1341-1349.

Wolf D.E., Maynard V.M., McKinnon C.A. and Melchior D.L. (1990). Lipid Domains in the Ram Sperm Plasma Membrane Demonstrated by Differential Scanning Calorimetry. *Proc. Natl. Acad. Sci. USA*. 87: 6893-6896

Wondji C. S. Coleman M., Kleinschmidt I., Mzilahowa T., Irving H. and Ndula M. (2012). Impact of pyrethroid resistance on operational malaria control in Malawi. *Proc. Natl. Acad. Sci. USA.* 109: 19063–19070.

Woodhead A.P., Thompson M.E., Chan K.K. and Stay B. (2003). Allatostatin in ovaries, oviducts, and young embryos in the cockroach Diploptera punctata. *J. Insect Physiol.* 49: 1103–1114.

Woolsey J. and Ingermann R. L. (2003). Acquisition of the potential for sperm motility in steelhead (*Oncorhynchus mykiss*): effect of pH on dynein ATPase. *Fish Physiol. Biochem.* 29: 47–56.

Yamagata K., Nakanishi T., Ikawa M., Yamaguchi R., Moss S.B. and Okabe M., (2002). Sperm from the calmegin-deficient mouse have normal abilities for binding and fusion to the egg plasma membrane. *Dev. Biol.* 250: 348-357.

Yamasaki N., Richardson R.T. and O'Rand M.G. (1995). Expression of the rabbit sperm protein Sp17 in COS cells and interaction of recombinant Sp17 with the rabbit zona pellucida. *Mol. Reprod. Dev.* 40: 48-55.

Yanagimachi R. (1994): In: Knobil, E., Neill, J.D. (Eds.), The Physiology of Reproduction. Raven Press, New York, 2nd Ed., pp. 189-317.

Yang Y., Cochran D.A., Gargano M.D., King I., Samhat N.K., Burger B.P., Sabourin K.R., Hou Y., Awata J., Parry D.A., Marshall W.F., Witman G.B. and Lu X. (2011) Regulation of flagellar motility by the conserved flagellar protein CG34110/Ccdc135/FAP50. Mol Biol Cell. 22:976–987.

Yang Y., and Lu X. (2011). Drosophila sperm motility in the reproductive tract. Biol Reprod. 84:1005–1015.

Yau M.K., Liu L. and Fairlie D.P. (2013). Toward Drugs for Protease-Activated Receptor 2 (PAR2). J Med. Chem. 56: 7477−7497

Yoshida-Komiya H., Tulsiani D., Hirayama T. and Araki Y. (1999). Mannose-binding molecules of rat spermatozoa and sperm-egg interaction. *Zygote. 7* (4): 335-346.

Zdarek J., Nachman R.J. and Denlinger D.L. (2000). Parturition hormone in the tsetse *Glossina morsitans*: activity in reproductive tissues from other species and response of tsetse to identified neuropeptides and other neuroactive compounds. *J. Insect Physiol.* 46: 213–219.

Zitta K., Werthheimer E. and Miranda P. (2006). Sperm N-acetylglucosaminidase is involved in primary binding to the zona pellucida. *Mol. Hum. Reprod*. *12* (9): 557-563.

Zylka M.J., Shearman L.P., Weaver D.R. and Reppert S.M. (1998). Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron*. 20: 1103–1110.

Zylka M.J., Shearman L.P., Levine J.D., Jin X., Weaver D.R. and Reppert S.M. (1998). Molecular analysis of mammalian timeless. *Neuron.* 21:1115–1122.

Figures

Figure 1.1A. Diagram of the three waveforms exhibited by *Culex* sperm.

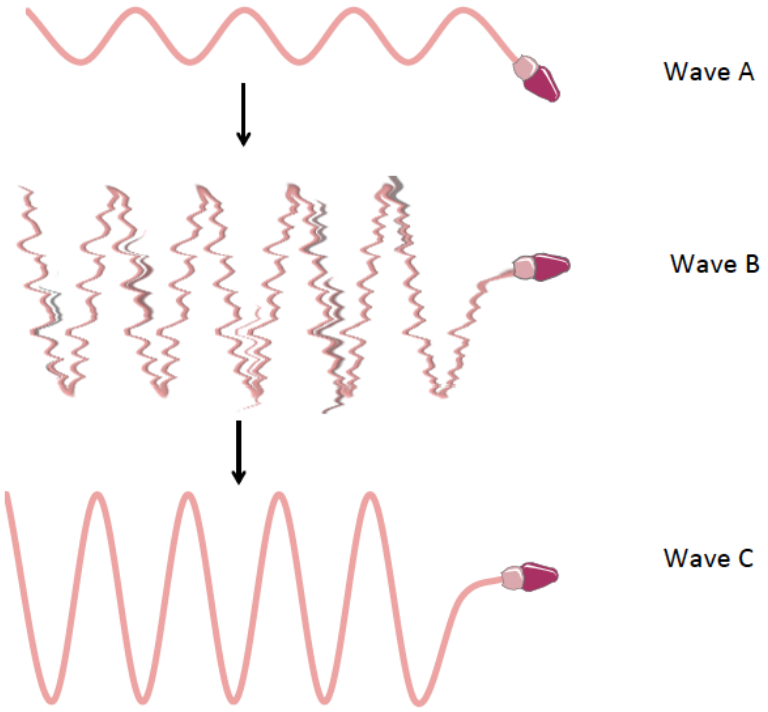


Figure 1.1B. Images of the three waveforms exhibited by *Culex* sperm. Taken from Thaler et al 2014.

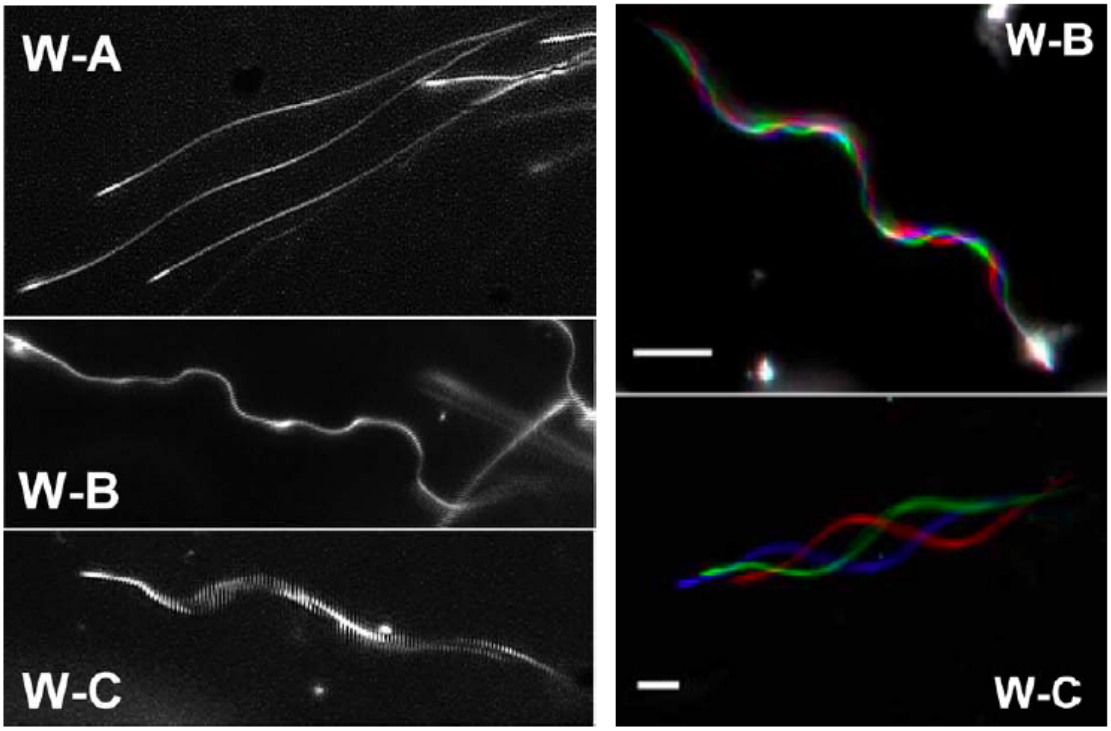


Figure 1.1C. Diagram of the structure of *Aquarius remigis* sperm. Taken from Miyata et al. 2010.

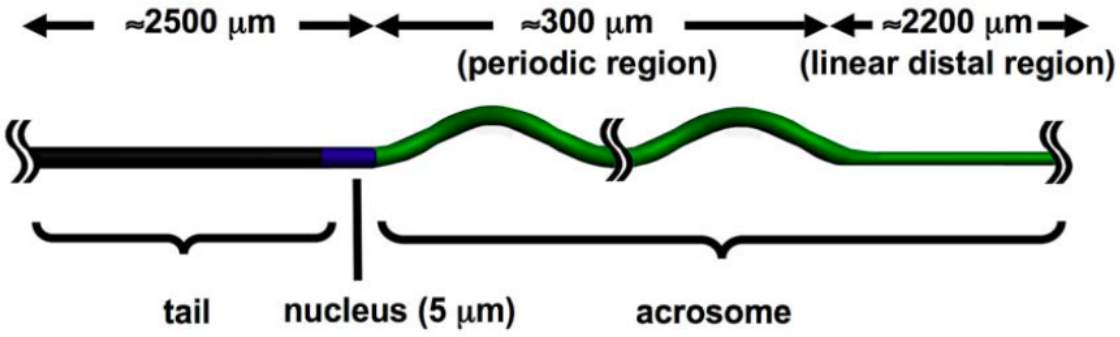


Figure 1.1D. Images of the structure of *Aquarius remigis* sperm. Taken from Miyata et al. 2010.

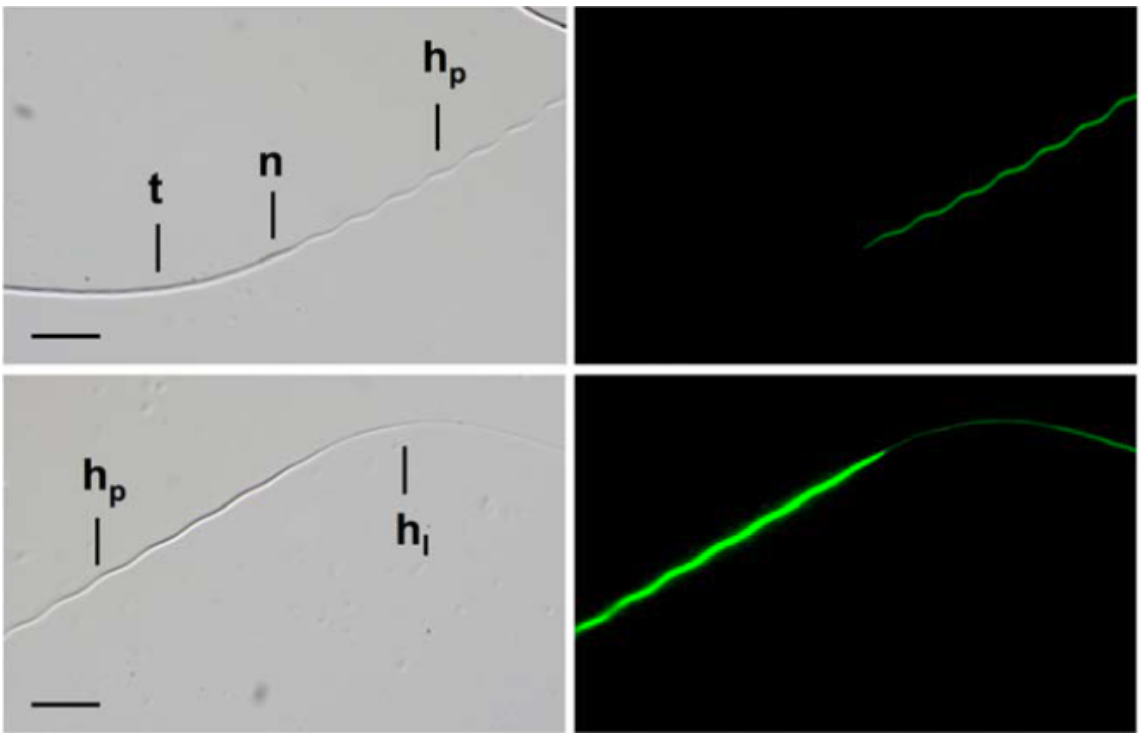


Figure 1.1E. Schematic illustration of Culex quinquefasciatus sperm motility regulation. Motility activators and inhibitors are shown. Taken from Thaler et al 2014.

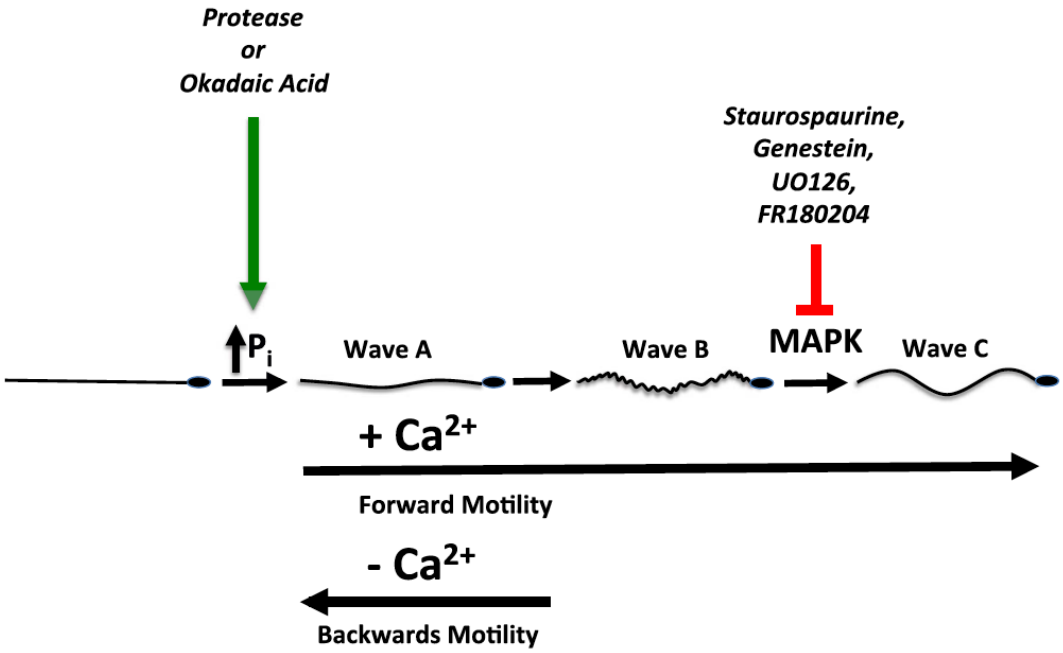


Figure 1.1F. Image of the 9+9+2 structure of an insect sperm axoneme from Dallai, 2014.

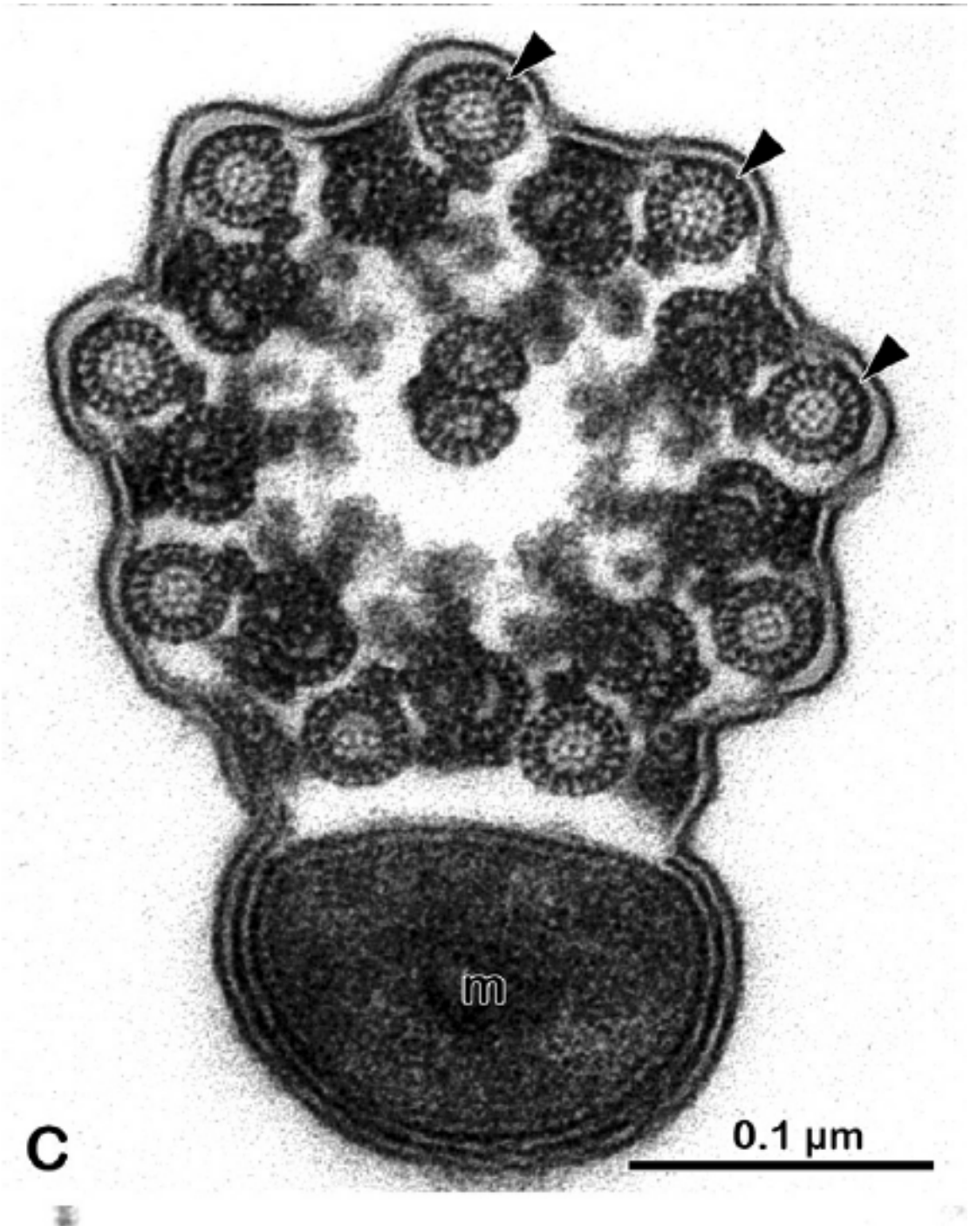


Figure 1.2. Diagram of the male reproductive tract of human. Image of the male reproductive tract was taken Servier Medical Art Reproduction kit (http://www.servier.com/Powerpoint-image-bank). Images were labeled.

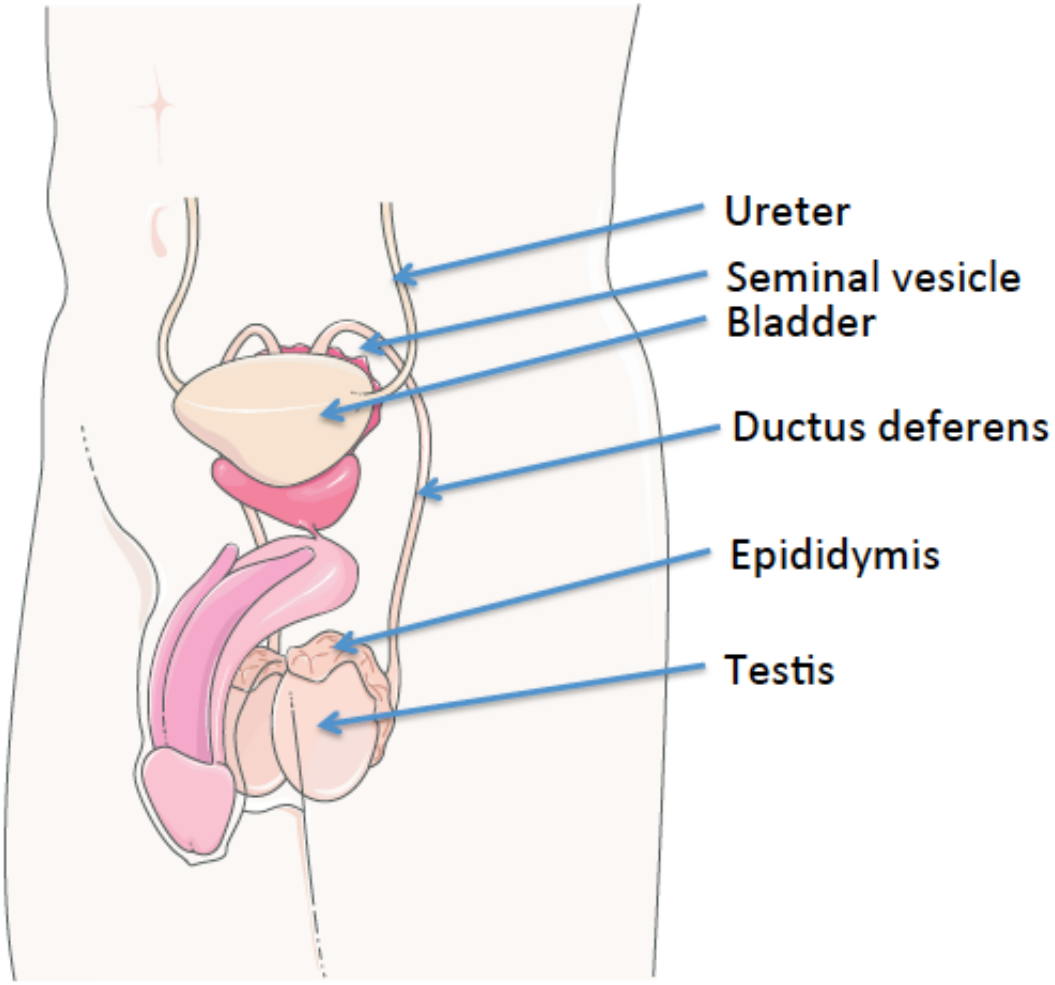
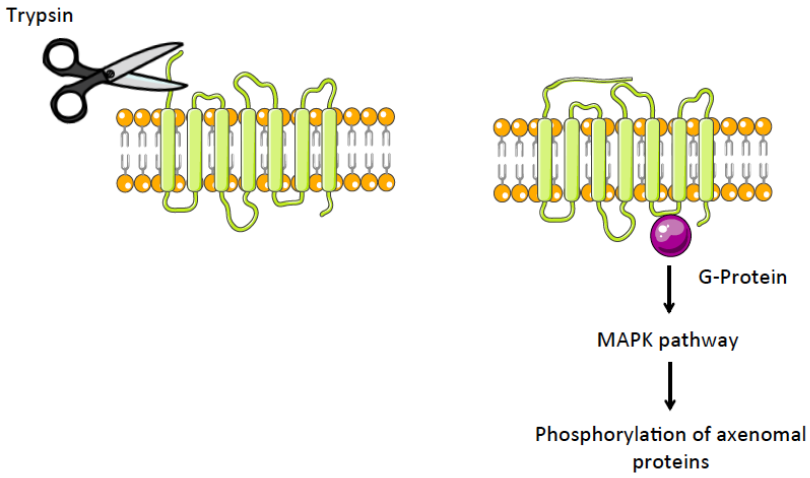


Figure 1.3. Diagram of PAR2-like activation of MAPK pathway via trypsin cleavage of an RSLIGRL sequence. PM stands for plasma membrane. PARs are GPCRs that are activated through the cleavage of a specific protease. Clip art was taken Servier Medical Art Reproduction kit (http://www.servier.com/Powerpoint-image-bank). Shapes were arranged to illustrate a trypsin activated PAR2-like pathway.



CHAPTER 2

Muscle Contractions in the Male Reproductive Tract of *Culex*

Muscle Contractions in the Male Reproductive Tract of *Culex*

Abstract

Muscle contractions are necessary for moving eggs from the oviduct to the site of fertilization in the female reproductive tract. We wanted to determine if muscle contractions in the male reproductive tract in a mosquito, *Culex pipiens*, provide a mechanism for sperm delivery to the female reproductive tract as well. We tested to see if muscular tissue was present in the male reproductive tract using the F-actin stain, rhodamine-phalloidin. While both accessory glands and seminal vesicles had F-actin, the muscle tissue in seminal vesicles was more organized. We next confirmed that increasing intracellular calcium was sufficient to induce muscular contractions in seminal vesicles, but not accessory glands, by treating them with the calcium ionophore, ionomycin. Several neurotransmitters were tested to see if they induced muscular contractions in the male reproductive tract. Acetylcholine induced contractions in both seminal vesicles and accessory glands, whereas nicotine was able to stimulate contractions only in seminal vesicles, supporting a role for nAChRs in sperm delivery from seminal vesicles. In contrast, pilocarpine (a muscarinic aceylcholine receptor agonist), serotonin, octopamine, or thapsigargin (a SERCA inhibitor), failed to stimulate contractions in either tissue. Further, neither m-3M3FBS, a PLC activator, or caffeine, a PDE inhibitor, caused muscle contractions. However, IBMX, a more potent PDE suppressor, did cause muscle contractions. These data suggest that an increase in cyclic nucleotides, but not the PLC pathway, is involved in the muscle contraction activation. Together, these data indicate that muscle contractions in the male reproductive tract are activated by stimulation of a

nicotinic acetylcholine receptor, are Ca^{2+} dependent and are initiated by an increase in cyclic nucleotides.

Introduction

In insects, the male transfers both sperm and accessory gland fluid to the female upon mating. Within the accessory gland fluid, for many insects, are compounds and proteins that modulate female sexual behavior or egg laying abilities (Cordero, 1995; Sirot et al., 2006) and compounds that modulate sperm behavior such as activators of sperm motility (Thaler et al., 2014).

Culex mosquitoes have paired testes that lead to paired seminal vesicles, which are in close proximity to the accessory glands. Sperm are produced in the testes and then moved to seminal vesicles for storage prior to mating. Sperm is not likely activated until exposure to accessory gland components (Thaler et al., 2014). This raises the question of how sperm are transported from male to female reproductive tract. A possible mechanism for sperm movement from the male to the female reproductive tract, prior to exposure to the accessory gland fluid, is through muscle contractions. Little is known about the musculature of seminal vesicles within *Culex* mosquitoes and knowledge of seminal vesicle musculature could provide insight into movement of sperm.

Muscles can provide structure, control movement, and move blood and other substances through the body. In mammals, there are two main types of muscle tissue: smooth and striated (including skeletal and cardiac). Both smooth and striated muscle require a signal in order to start contracting (Webb, 2003; Berchtold et al., 2000; Lehman et al., 1994). Nicotinic acetylcholine receptors initiate contractions in striated skeletal muscle but muscarinic acetylcholine receptors initiate contractions in smooth muscles (Guyton and Hall, 2006; Hoffman and Taylor, 2001). Smooth muscle receives neural

innervation from the autonomic nervous system (Webb, 2003). In smooth muscle, contractions are stimulated by a change in membrane potential. The myosin light chain kinase phosphorylates myosin light chains allowing myosin and actin to interact (Webb, 2003). The energy is provided by the breakdown of ATP by myosin ATPase allowing the myosin crossbridges to cycle with actin resulting in contraction (Webb, 2003). Therefore, regulation of smooth muscle contraction relies heavily on the phosphorylation state of the myosin light chains (Webb, 2003). However, this does not suggest that calcium plays no role in smooth muscle contraction. In both striated and smooth muscle, intracellular calcium increases via intracellular stores such as the sarcoplasmic reticulum and extracellularly through calcium channels (Hill-Eubanks et al., 2011; Webb, 2003). Calcium then interacts with calmodulin to activate myosin light chain kinase that then phosphorylates myosin light chains (Webb, 2003). Smooth muscle cells are connected by gap junctions, which are intracellular channels formed by connexin proteins (Goodenough et al., 1996) that permit small molecules (such as calcium) to move freely from cell to cell. Electrically excitable cells require an ability to share ions with adjacent cells through low-resistance pathways in order to synchronize groups of cells to obtain a coordinated electrical and mechanical output (Weidman, 1952; Furshpan and Potter, 1957). In smooth muscle, the connections between cells that are formed by gap junctions increases synaptic transmission speed and allows for muscle contraction to be coordinated with all cells that make up the tissue.

In striated muscle, tropomyosin is shifted away from myosin binding sites on actin by troponin after calcium has bound to troponin. When tropomyosin shifts, myosin

binding sites are exposed, which allows myosin to bind actin initiating the cross-bridge cycle (Lehman et al., 1994). The cross-bridge cycle pulls actin filaments toward the center of the sarcomere resulting in muscle fiber shortening (Lehman et al., 1994). In striated muscle contractions, either GPCR pathways or ionotropic receptors that are activated by neurotransmitters such as acetylcholine, serotonin or octopamine stimulate the calcium release necessary for muscle contractions to occur (Steers, 1994; Chen et al., 2014; Nichols and Nichols, 2008).

Unlike mammals, insects do not have smooth muscle tissue (Elzinga, 2003; Klowden, 2007). Insects have two forms of striated muscle: visceral and skeletal (Klowden, 2007). Visceral muscle surrounds the viscera but does not attach to the body wall, whereas skeletal muscle is anchored to exoskeleton at both ends and skeletal parts move relative to each other (Klowden, 2007). Skeletal muscle is further divided into synchronous and asynchronous muscle based on nervous regulation (Klowden, 2007). Synchronous muscles contract in synchrony with the innervating motor neurons, whereas asynchronous muscles contract do not contract in synchrony with the innervating motor neurons (Klowden, 2007).

Neurotransmitters are involved in regulating muscle contractions in reproductive processes such as ovulation and egg movement. Proctolin, a pentapeptide, is known to stimulate oviduct contractions in many species of insects including *Leucophaea maderae*, *Tabanus sulcifrons*, *Locusta migratoria* and *Rhodnius prolixus* (Cook and Maeola, 1978, Holman and Cook, 1979, Lange et al., 1986 and Lange, 1990). In *L. migratoria*, crustacean cardioactive peptide is also known to increase muscle contractions (Donini et

al., 2001). The FMRFamide-like peptide (FLP) families are known to stimulate oviduct contractions in *R. prolixus* and *L. migratoria* (Sedra and Lange, 2013; Peeffe et al., 1993). Despite the plethora of data available on effects of neurotransmitters in insect female reproductive muscle contraction, there is a dearth of information available on involvement of neurotransmitters in male reproductive muscle contractions. Fertilization, at any step, including muscle contractions of the reproductive tract, represents a potential target for developing mosquito control methods. However, the design of targeted and effective pesticides requires an understanding of the specific mechanisms that are unique to mosquito reproduction, in this case, muscular contractions that are involved in sperm delivery through the male reproductive tract. In this study, we report that F-actin staining is consistent with striated muscle in the seminal vesicles and that nictonic acetylcholine receptors are involved in muscular contractions. We demonstrate that nicotine and acetylcholine, but not pilocarpine, octopamine or serotonin, stimulate muscle contractions. We also utilize several pharmacological agents to determine that an increase in cyclic nucleotides but not the PLC pathway is involved in the male reproductive tract muscle contractions. Together, these experiments provide insight into the mechanism of activation and propagation of muscle contractions within the *Culex* mosquito.

Materials and Methods

Chemicals and reagents

Pilocarpine, serotonin, nicotine, acetylcholine and octopamine were purchased from Tocris Bioscience (R and D Systems). Rhodamine-phalloidin and rhodamine bungarotoxin were purchased from Invitrogen. All other reagents were obtained from Sigma-Aldrich or Fisher Scientific.

Dissection of the male reproductive tract

Male *Culex pipiens* were obtained from a colony maintained by Dr. Edward Platzer in the Department of Biology at the University of California, Riverside. Mosquitoes were housed individually in 1.25 ounce solo cups (product #P125, Solo Cup Company) with lids (product #PL1, Solo Cup Company). Dilute sugar water was added to each cup to humidify the chamber and provide food. Mosquitoes were anesthetized by inserting a small piece of chloroform-soaked cotton into the cup. Subsequently, the male reproductive tract was dissected from the mosquitoes as previously described (Thaler et al., 2014).

Phalloidin Staining

Male reproductive tracts were placed on slides coated in 1% polyethyleneimine (PEI), fixed in 4% formaldehyde for 10 min, washed in TBS for 10 min and subsequently immersed in 1% Triton X-100 to permeabilize the tissue in TBS for 30 min. After the samples were permeabilized, they were blocked using 5% Bovine Serum Albumin (BSA) in TBS for 15 min and washed in TBS alone twice for 15 min. Subsequently, the samples were incubated for 30 min in 5.0×10^{-4} mg/mL rhodamine phalloidin (Invitrogen) in TBS.

Samples were then washed in 1% BSA in TBS for 15 min and, subsequently, twice more in TBS alone for 15 min. Samples were incubated in 100 μg/mL Hoechst for 15 min and washed washed twice in TBS for 15 min. Lastly, a drop of VectaShield (Vector Labs) was added and the samples were observed using a confocal microscope. A Leica TCS SP2 UV confocal microscope (Leica Microsystems, Heidelberg GmbH, Wetzlar, Germany) was used for fluorescence imaging. All images were collected using a 40x water immersion objective and sequential scan mode to eliminate fluorescence bleed through.

Bungarotoxin Staining

Slides were coated in 1% polyethyleneimine (PEI), fixed in 4% formaldehyde for 10 min, washed in TBS for 10 min and subsequently immersed in 1% Triton X-100 (Bio-Rad, Richmond, CA) in TBS for 30 min. After the samples were permeabilized, they were blocked using 5% Bovine Serum Albumin (BSA) in TBS overnight and washed in TBS alone, twice, for 15 min. After blocking, the samples were incubated overnight in 125 nM bungarotoxin-TRITC (Invitrogen) in TBS. As a control, samples were treated overnight with 500 μM nicotine as well as simultaneously treated with Bungarotoxin. Next, samples were washed in 1% BSA in TBS for 15 min and, subsequently, twice more in TBS alone for 15 min. Samples were then incubated in 1 μg/mL Hoechst for 15 min and washed washed twice in TBS for 15 min. Lastly, a drop of VectaShield (Vector Labs) was added and the slides were observed using a confocal microscope (Leica TCS SP2 UV).

Muscle contraction Assays

The testes, seminal vesicles and accessory glands were dissected out of a male mosquito, placed in imaging dishes in insect Ringer (2.53 mM $CaCl_2$, 110 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 2 mM KH_2PO_4, 2 mM Na_2HPO_4, 1 mM glucose, 20 mM hepes, pH 7.2), or in calcium free insect Ringer (1 mM EGTA, 110 mM NaCl, 5 mM KCl, 1.2 mM$MgCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 2 mM KH_2PO_4, 2 mM Na_2HPO_4, 1 mM glucose, 20 mM hepes, pH 7.2), as indicated in the results. Samples were treated with either 10 μM ionomycin (Liu and Hermann, 1978), 1 μM 5-hydroxytryptamine (Cook et al., 1978), 1 μM acetylcholine (Kerkut et al., 1965), 10 μM nicotine (O'Connor et al., 1965), 10 μM pilocarpine (Buschges et al., 1995), 100 μM octopamine (Middleton et al., 2006), 1 μM m-3M3FBS (Bae et al. 2003), 20 mM caffeine (Chen et al., 2005), or 20 μM thapsigargin (Hosoki and Iijima, 1995). Concentrations were determined by concentrations found in the literature. Muscle contractions were observed at 20X magnification using an Axiovert 10 (Zeiss) and images were recorded with Simple PCI (Hamamatsu, Corp., Bridgewater, NJ) using a Hamamatsu CCD camera. The contrast and brightness were adjusted on the images.

Results

Phalloidin Staining

A small region near the vas deferens of the testis displayed phalloidin staining (Figure 1). In contrast, the seminal vesicles and accessory glands showed phalloidin staining throughout (Figure 2 and 3). There were clear striations throughout the seminal vesicles that become more concentrated near the apical end (Figure 2). The striations in the accessory glands were organized in linear arrays throughout the entire organ (Figure 3). Coiling of the striations was also apparent at the tip of the accessory glands (Figure 3). Taken together, these data indicate that while the testes do not have substantial muscle tissue present, both the accessory glands and the seminal vesicles are muscular.

Ionomycin treatments

When treated with ionomycin in the absence of calcium, no contractions in the male reproductive tract were observed. In contrast, when the male reproductive tract was treated with ionomycin in the presence of calcium, the seminal vesicles contracted in a rhythmic fashion. These data indicate that the male reproductive tract, and specifically the seminal vesicles, can undergo muscle contractions *in vitro*, and that elevating intracellular Ca^{2+} is sufficient to initiate those contractions.

Neurotransmitter treatments

When treated with the neurotransmitters octopamine or 5-hydroxytryptamine (5-HT) no muscle contractions were elicited suggesting that neither a serotonin receptor nor

octopamine receptor are present on seminal vesicles. However, when treated with acetylcholine, muscle contractions were elicited in both seminal vesicles and accessory glands suggesting that activation of an acetylcholine receptor is sufficient to initiate muscular contractions and that Ca^{2+} is mobilized from an intracellular store. Normally in nicotinic acetylcholine receptors, Ca^{2+} is mobilized when two acetylcholines bind to a nicotinic acetylcholine receptor resulting in a conformational change in order to open an ion channel. When a pore opens at the neuromuscular junction, there is an influx of sodium and calcium ions causing a depolarization and excitation of muscle cells, which elicits muscle contractions.

To determine if the receptor is a muscarinic or nicotinic acetylcholine receptor, we treated the male reproductive tract with pilocarpine or nicotine, respectively. While pilocarpine did not stimulate contractions, nicotine was a potent stimulus for muscle contractions in the seminal vesicles indicating that a nicotinic, not muscarinic, receptor is present on the seminal vesicles. In sum, nicotinic receptors are present on the seminal vesicles in the mosquito, whereas octopamine or 5HT receptors were not present on any part of the male reproductive tract.

α-Bungarotoxin Staining

Nicotinic acetylcholine receptors (nAChR) are ligand gated pentameric ion channels. Mammals express 16 different subunits (α1–7, α9–10, β1–4, δ, ε, γ). Some of the channels formed by these subunits are homomeric complexes, while others are heteromeric and have distinct pharmacological properties (Lindstrom, 1995; Leonard and

Bertrand, 2001; Le Novere and Changeux, 1995). The most prevalent mammalian combinations of nAChR subunits are shown in Table 2. Within insects, *Drosophila melanogaster*, *Anopheles gambiae*, *Apis mellifera*, *Bombyx mori*, and *Tribolium castaneum* possess 10 to 12 nicotinic acetylcholine receptor genes (Jones and Satelle, 2010), while *Culex* possess three subunits: α7 (6 proteins), α4 or α2 (2 proteins) and β2 (2 proteins)—annotated in the NCBI website (Table 2). α-Bungarotoxin does not successfully bind to the *Culex* male reproductive tract as evidenced by a lack of specific TRITC signal coming from the male reproductive tract when incubated with α-bungarotoxin-TRITC (Figure 5). A negative result does not indicate absence of nicotinic receptors, because multiple subtypes of nicotinic receptors with various binding affinities to α-bungarotoxin (Table 2). The lack of α-bungarotoxin binding suggests that the nicotinic receptor is a subtype that may not interact with α-bungarotoxin.

Pathway activator treatments

When treated with m-3M3FBS neither seminal vesicles nor accessory glands contract (Table 3), indicating that the PLC pathway is not involved in activating contractions. Caffeine did not stimulate contractions, but IBMX did stimulate contractions (Table 3), indicating that an increase in cyclic nucleotides is sufficient to activate contractions. When the male reproductive tract was treated with the SERCA inhibitors thapsigargin or cyclopiazonic acid, there were no contractions; however, when treated with acetylcholine and then subsequently treated with a high concentration of

ryanodine, contractions were inhibited (Table 4). These data indicate that a thapsigargin-insensitive calcium store is likely to be the source of calcium.

Discussion

The accessory glands and seminal vesicles of *Culex* mosquitoes contract rhythmically, which is presumably to aid in movement of sperm and seminal fluid (from accessory glands) to the female through the ejaculatory duct. *Culex* testes, however, do not contract. In a species of earwig, *E. annulipes*, male accessory glands and seminal vesicles contracted in response to various treatments, but the testes did not (Rankin et al., 2009). This result is similar to what we observed in the *Culex* male reproductive tract and is consistent with the presence of F-actin in the accessory glands and seminal vesicles. Additionally, *E. annulipes* testes affected the contraction rate of the rest of the male reproductive tract: when the testes were removed, the contractions in the other organs were reduced (Rankin et al., 2009). Likewise, when the testes are removed from *Culex*, stimulating contractions in the other tissues was not possible. In two species of beetles, *Tenebrio molitor* and *Zophobas atratus*, neuropeptides are able to stimulate muscle contractions in the male ejaculatory duct (Marciniak and Rosinski, 2010). Lastly, a species of moth, *Lymantria dispar*, undergoes contractions of the upper vas deferens associated with testicular sperm release that is regulated by circadian cycles (Giebultowicz et al., 1995). Based on these observations, it is possible that circadian rhythms in insects, including *Culex* mosquitoes, may regulate muscle contractions by regulating neuropeptide release.

For many insects, muscle contractions are known to be involved in female reproduction (Cook and Maeola, 1978, Holman and Cook, 1979, Lange et al., 1986; Lange, 1990). In female insects, muscle contractions are involved in egg movement and

ovulation and the effect of neurotransmitters on reproductive muscle contractions is well studied. In contrast, however, in the male reproductive tract, less is known regarding the role of neurotransmitters where muscle contractions may serve to move not only sperm but also accessory gland components to the female. There are several components that could potentially be transferred to the female reproductive tract through male reproductive muscle contractions. Some of these include compounds that reduce female receptivity (Chen et al., 1988; Aigaki et al., 1991; Kubli, 1992, 1996; Moshitzky et al., 1996; Nakayama et al., 1997; Soller et al., 1997, 1999; Ottiger et al., 2000), or promote ovulation (Rubenstein and Wolfner, 2013) or oviposition (Gillott, 1988; Gillott, 2003; Kubli, 2003, Chapman and Davies, 2004; Ravi Ram and Wolfner, 2007).

Culex male reproductive tracts undergo contractions when exposed to ionomycin in the presence of calcium indicating that calcium entry into the cell is sufficient to stimulate contractions. These muscle contractions can be initiated in the presence of neurotransmitters including acetylcholine and nicotine but not pilocarpine supporting a role for a nicotinic acetylcholine receptor in the male reproductive tract. In contrast, neither octopamine nor serotonin is capable of activating muscle contractions. These results differ from many female insect reproductive tracts where octopamine is known to stimulate ovarian and spermathecal muscle contractions and oviduct relaxation (Cook and Peterson, 1989; Lange and Tsang, 1993; Cook and Wagner, 1992; Clark and Lange, 2003).

A calcium ionophore, ionomycin, is sufficient to activate muscle contractions. Taken together, this result suggests that calcium is necessary and sufficient for activating

contractions. The SERCA inhibitors thapsigargin and cyclopiazonic acid did not stimulate muscle contractions. However, when muscle contractions were stimulated with acetylcholine, the contractions could be inhibited with high concentrations of ryanodine. High concentrations of ryanodine lock the receptor into a closed state consistent with inhibition of muscle contractions (Dondas et al., 2009; Xu et al., 1998). The PLC activator, m-3M3FBS does not activate muscle contractions, suggesting that the PLC pathway is not involved in activating muscle contractions in the male reproductive tract.

The phosphodiesterase inhibitor, caffeine, did not activate muscle contractions but a different phosphodiesterase inhibitor, IBMX, did activate muscle contractions, indicating that an increase in cyclic nucleotides is important for activating muscle contractions. Three pathways that could be activated by inhibiting phosphodiesterase are the protein kinase A, protein kinase C or protein kinase G (PKA, PKC or PKG) pathways. The protein kinase pathways could potentially activate calcium release through the ryanodine receptor. Based on these data, it is likely that a nicotinic acetylcholine receptor (nAChR) is activated, causing an influx of calcium into the system leading to seminal vesicle muscle contractions (Figure 6).

In insects, there are usually 10-12 nAChR genes while humans and nematodes have 16 and 29 nAChR genes, respectively (Jones and Sattelle, 2010; Millar and Gotti, 2009; Littleton and Ganetzky, 2000). Antihelmetics such as levamisole, pyrantel and morantel target nAChRs present in the nematode body wall (Jones and Sattelle, 2010; Brown et al., 2006). Despite having fewer genes that code for nAChR, the central nervous system of insects is rich in nAChR (Jones and Sattelle, 2010). In fact, a major

class of insecticides, neonicitinoids, target insect nAChRs and are selective for insect nAChRs over mammalian nAChRs (Jones and Sattelle, 2010; Tomizawa and Casida, 2003; Nishiwaki et al., 2003). However, previous literature suggested that nAChRs do not act in the neuromuscular junctions of insects (Faeder et al., 1970). In cockroaches, acetylcholine can activate cardiac muscle contractions (Collins and Miller, 1977). There is evidence that nicotine can stimulate cardiac ganglia in the cockroach heart (Srivinas et al 1984) suggesting that nicotinic receptors are present at the neuromuscular junction of the cockroach heart. In *Culex pipiens,* I have found evidence of a nicotinic receptor present in the peripheral nervous system suggesting it is possible that nicotinic receptors are present at the neuromuscular junctions of *Culex pipiens* seminal vesicles.

References

Aigaki T., Fleischmann I., Chen P.S. and Kubli E. (1991). Ectopic expression of sex peptide alters reproductive behavior of female *D. melanogaster. Neuron*. 7: 557–563.

Bae Y.S., Lee T.G., Park J.C., Hur J.H., Kim Y., Heo Kyon, Kwak J.Y., Suh P.G. and Ryu S.H. (2003) Identification of a compound that directly stimulates phospholipase C activity. *Mol.Pharmacol*. 63:1043.

Chapman T. and Davies S.J. (2004). Functions and analysis of the seminal fluid proteins of male *Drosophila melanogaster* fruit flies. *Peptides* 25: 1477–1490.

Chen P.S. (1991). Biochemistry and molecular regulation of the male accessory gland secretions in *Drosophila* (Diptera). *Annals Soc Ent* Fr 27: 231–244.

Collins C., Miller T. (1977). Studies on the action of biogenic amines on cockroach heart. *J. Exp. Biol. 67*: 1–15.

Cook B.J. and Maeola S. (1978). The oviduct musculature of the horsefly, *Tabanus sulcifrons*, and its response to 5-hydroxytryptamine and proctolin *Physiol. Entomol*. 3: 273–280

Cook B.J. and Peterson T. (1989). Ovarian muscularis of the stable fly *Stomoxys calcitrans* – Its structural, motile, and pharmacological properties. *Arch Insect Biochem Physiol*. 12:15-30.

Cook B.J. and Wagner R.M. (1992). Some pharmacological properties of the oviduct muscularis of the stable fly, *Stomoxys calcitrans*. *Comp Biochem Physiol C.* 102:273-280.

Clark J. and Lange A.B. (2003). Octopamine modulates spermathecal muscle Contractions in *Locusta migratoria*. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 189:105-114.

Cordero C. (1995). Ejaculate substances that affect female insect reproductive physiology and behavior: honest or arbitrary traits? *J. Theor. Biol.* 174: 453–461.

Donini, Agricola and Lange. (2001). Crustacean cardioactive peptide is a modulator of oviduct contraction in *Locusta migratoria*. *J. Insect Physiol.* 47: 277–285

Elzinga R.J. (2003). *Fundamentals of entomology* (6th ed.). Upper Saddle River, NJ: Prentice Hall

Faeder I.R., O'Brien R.D. and Salpeter M.M. (1970). A Re-investigation of Evidence for Cholinergic Neuromuscular Transmission in Insects. *J. Exp. Zool*., 173: 187-202.

Gillott C. (1988). Arthropoda-Insecta. In: Adiyodi K.G. and Adiyodi R.G. (Eds.), Reproductive Biology of Invertebrates, Vol. III. Accessory Sex Glands. Wiley, New York, pp. 319–471.

Giebultowicz J.M., Blackburn M.B., Thomas-Laemont P.A., Weyda F. and Raina A.K. (1995). Daily rhythm in myogenic contractions of vas deferens associated with sperm release cycle in a moth. *J Comp Physiol A*. 178: 629-636

Gillott C. (2003). Male accessory gland secretions: modulators of female reproductive physiology and behavior. *Ann Rev Entom* 48, 163–184.

Holman and Cook. (1979). Evidence of proctolin and a second myotropic peptide in the cockroach, *Leucophaea maderae*, determined by bioassay and HPLC analysis. *Insect Biochem.* 9:149–154.

Jones A. K. and Sattelle D. B. (2010). Diversity of Insect Nicotinic Acetylcholine Receptor Subunits. In S. H. Thany, and S. H. Thany (Ed.), *Insect Nicotinic Acetylcholine Receptors.* New York, New York, USA: Landes Bioscience and Springer Science+Business Media, LLC.

Klowden, M. J. (2007). Physiological systems in insects. New York: Academic Press.

Kubli E. (1992). My favorite molecule: the sex peptide. *Bioessays* 14: 779–784.

Kubli E. (1996). The Drosophila sex-peptide: a peptide pheromone involved in reproduction. *Adv Devel Biochem* 4: 99–128.

Kubli E. (2003). Sex peptides: seminal peptides of the Drosophila male. *Cellular and Molecular Life Sciences* 60, 1689–1704.

Lange, Orchard and Adams. (1986). Peptidergic innervation of insect reproductive tissue: the association of proctolin with oviduct visceral musculature. *J. Comp. Neurol.* 254: 279–286

Lange. (1990). The presence of proctolin in the reproductive system of *Rhodnius prolixus*. *J. Insect Physiol.* 36: 345–351

Lange A.B., Tsang P.K.C. (1993). Biochemical and physiological effects of octopamine and selected octopamine agonists on the oviducts of *Locusta migratoria*. *J Insect Physiol* 39:393-400.

Lindstrom J. M. in *Hand Book of Receptors and Channels: Ligand- and Voltage-Gated Ion Channels* (ed. North, A.) 153-175 (CRC Press, Boca Raton, Florida, 1995)

Littleton J.T. and Ganetzky B. (2000). Ion channels and synaptic organization: analysis of the *Drosophila* genome. *Neuron* 26:35-43.

Leonard S. and Bertrand D. (2001). Neuronal nicotinic receptors: from structure to function. *Nicotine Tob. Res.* **3**: 203-223.

Le Novere N. and Changeux J.P. (1995). Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. *J. Mol. Evol.* **40**: 155-172.

Liu and Hermann. (1978). Characterization of ionomycin as a calcium ionophore. *J.Biol.Chem*. 253: 5892.

Marciniak P. and Rosinski G. (2010). Comparison of proctolin and FMRFamide actions on the motility of male and female beetle reproductive tracts. *IRD* 54: 1-6

Millar N.S. and Gotti C. (2009). Diversity of vertebrate nicotinic acetylcholine receptors. *Neuropharmacology*. 56(1): 237-46

Moshitzky P., Fleischmann I., Chaimov N., Saudan P., Klauser S. and Kubli E. (1996). Sex-peptide activates juvenile hormone biosynthesis in the *Drosophila melanogaster* corpus allatum. *Arch Insect Bioch Physiol* 32: 363–374.

Nakayama S., Kaiser K., Aigaki T. (1997). Ectopic expression of sex peptide in a variety of tissues in *Drosophila* females using the P[GAL4] enhancer-trap system. *Mol Gen Genet* 254: 449–455.

Nässel and Winther. (2010). *Drosophila* neuropeptides in regulation of physiology and behavior. *Prog. Neurobiol*. 92: 42–104.

Nishiwaki H., Nakagawa Y. and Kuwamura M. (2003). Correlations of the electrophysiological activity of neonicotinoids with their binding and insecticidal activities. *Pest Manag Sci* 59:1023-1030.

Orchard, Lange and Bendena. (2001). FMRFamide-related peptides: a multifunctional family of structurally related neuropeptides in insects. *Adv. Insect Physiol.* 28: 267–329.

Ottiger M., Soller M., Stocker R.F. and Kubli E. (2000). Binding sites of *Drosophila melanogaster* sex peptide pheromones. *J Neurobiol* 44: 57–71.

Peeff, Orchard and Lange. (1993). The effects of FMRFamide-related peptides on an

insect (*Locusta migratoria*) visceral muscle. *J. Insect Physiol*. 39: 207–215.

Rankin S.M., TeBrugge V.A., Murray J.A., Schuler A.M. and Tobe S.S. (2009). Effects of selected neuropeptides, mating status and castration on male reproductive tract movements and immunolocalization of neuropeptides in earwigs. *Comparative Biochemistry and Physiology* **152**: 83–90.

Ravi Ram K. and Wolfner M.F., (2007). Sustained post-mating response in *Drosophila melanogaster* requires multiple seminal fluid proteins. *PLoS Genetics* 3, 2428–2438.

Rubenstein C.D. and Wolfner M.F. (2013). *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proc Natl Acad Sci*. 110 (43): 17420-17425

Sedra and Lange. (2013). The female reproductive system of the kissing bug, *Rhodnius prolixus*: arrangement of muscles, distribution and myoactivity of two endogenous FMRFamide-like peptides. *Peptides.* 53: 140–147

Sirot L.K., Lapointe S.L., Shatters R. and Bausher M., (2006). Transfer and fate of seminal fluid molecules in the beetle, *Diaprepes abbreviatus*: implications for reproductive biology in a pest species. *J. Insect Physiol*. 52: 300–308.

Soller M., Bownes M. and Kubli E. (1997). Mating and sex peptide stimulate the accumulation of yolk in oocytes of *Drosophila melanogaster*. *Eur J Biochem* 243: 732–738.

Soller M., Bownes M. and Kubli E. (1999). Control of oocyte maturation in sexually mature *Drosophila* females. *Dev Biol* 208: 337–351.

Srinivas P., Rama Rao G.D., Rao G.V., and Purushotham Rao A. (1984). Cardiac Stimulation of Brucine in Cockroach, Periplaneta Americana, L. *Ind. J. Physiol. Pharmac* 29 (1): 59-61.

Tomizawa M. and Casida J.E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol*. 48:339-364.

Figures and Tables

Figure 2.1. Rhodamine–phalloidin labeling of testes. Panel A, Hoescht staining (blue) of nuclei. Panel B, rhodamine phalloidin staining (red) of actin. Panel C, merged images. Only a small region near the vas deferens of the testis displayed phalloidin staining indicating that this tissue does not have muscular tissue throughout. N=2.

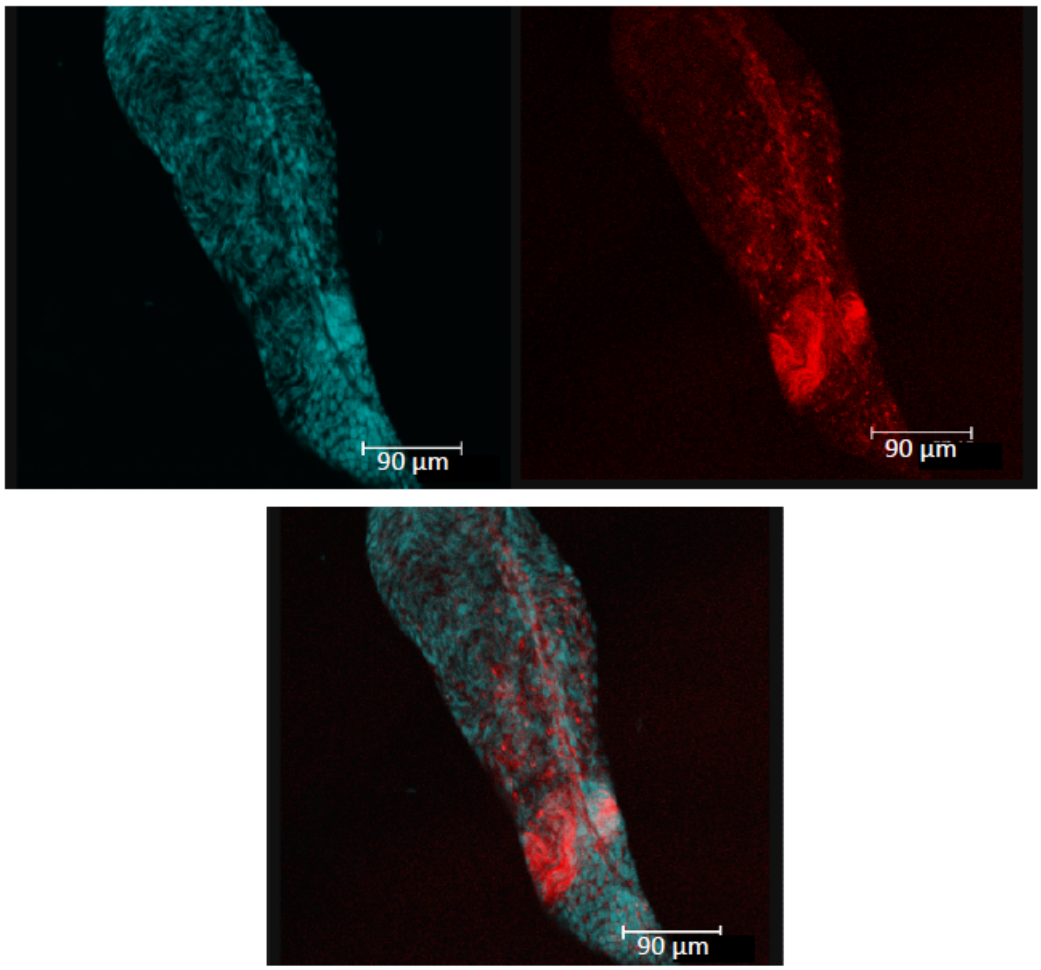


Figure 2.2. Rhodamine–phalloidin labeling of seminal vesicles. Panel A, Hoescht staining (blue) of nuclei. Panel B, rhodamine phalloidin staining (red) of actin. Panel C, merged images. There were clear striations throughout the seminal vesicles but these became more concentrated near the apical end, suggesting that the seminal vesicles are muscular. N=2.

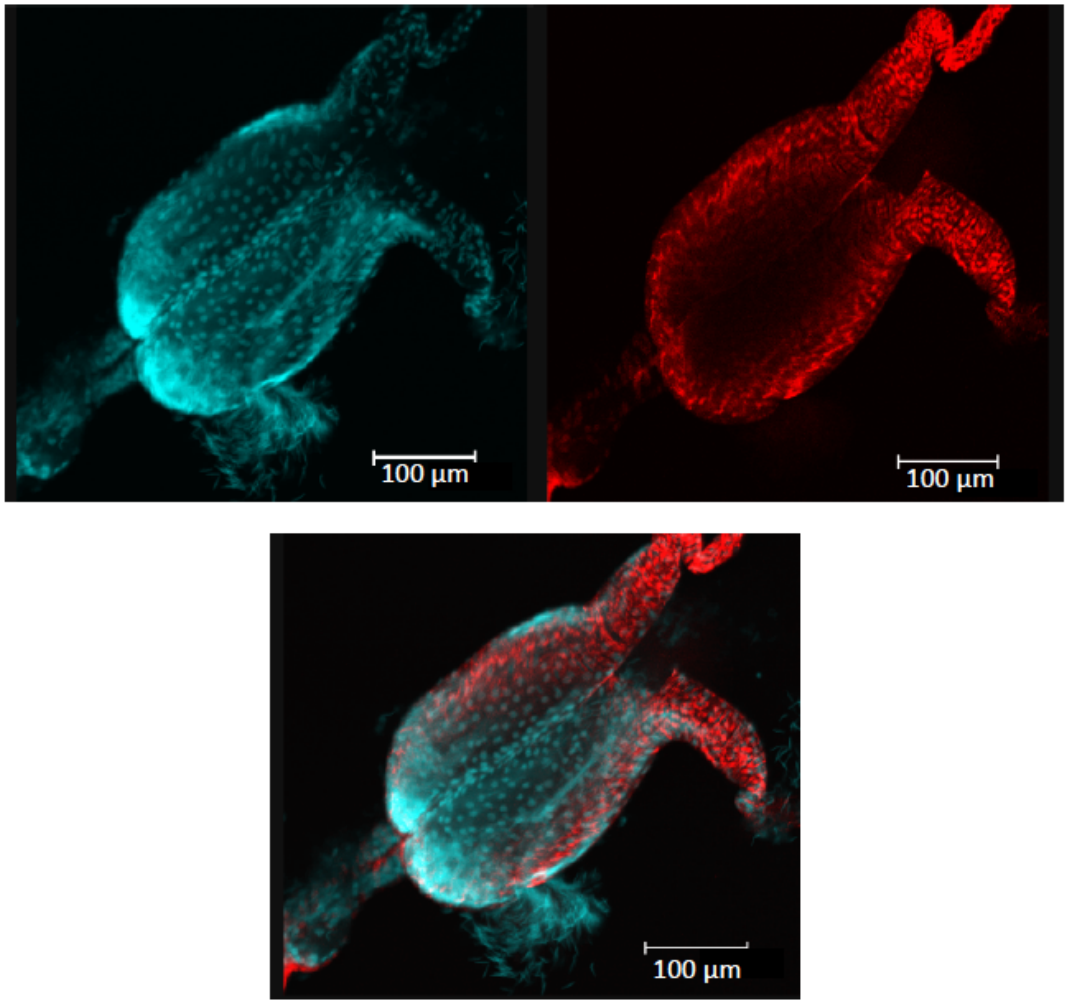


Figure 2.3. Rhodamine–phalloidin labeling of accessory glands. Panel A, Hoescht staining (blue) of nuclei. Panel B, rhodamine phalloidin staining (red) of actin. Panel C, merged images. The striations in the accessory glands were organized and linear throughout the entire organ. Coiling of the striations was also apparent at the tip of the accessory glands. This result suggests that the accessory glands are muscular. N=2.

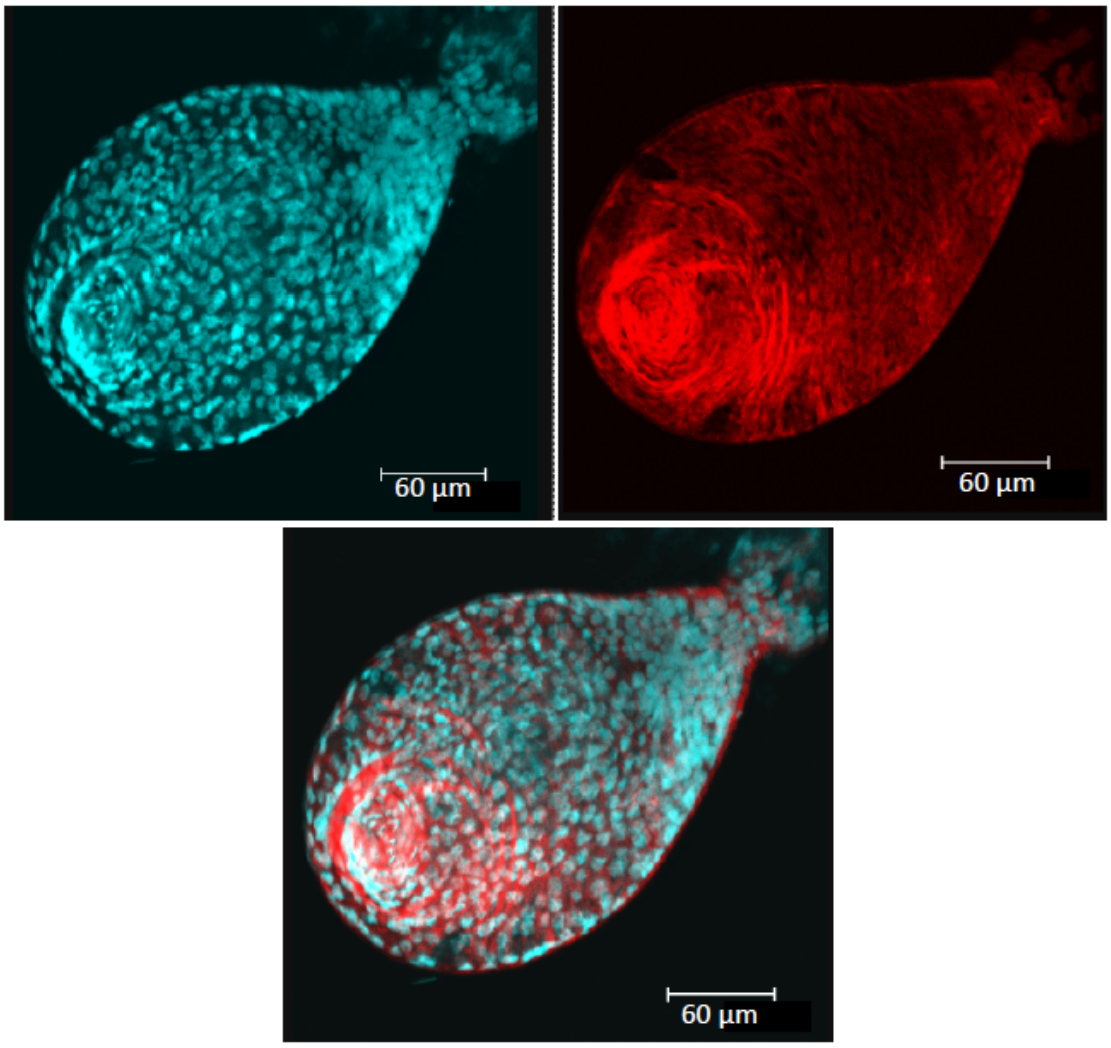


Figure 2.4. When ionomcycin is applied to the male reproductive tract, the seminal vesicles contract, on average, ~every 5.4 seconds. This graph shows an analysis of the ionomycin treatment where each contraction was counted. The contracted state is represented as one and the relaxed state is represented as zero. N=1.

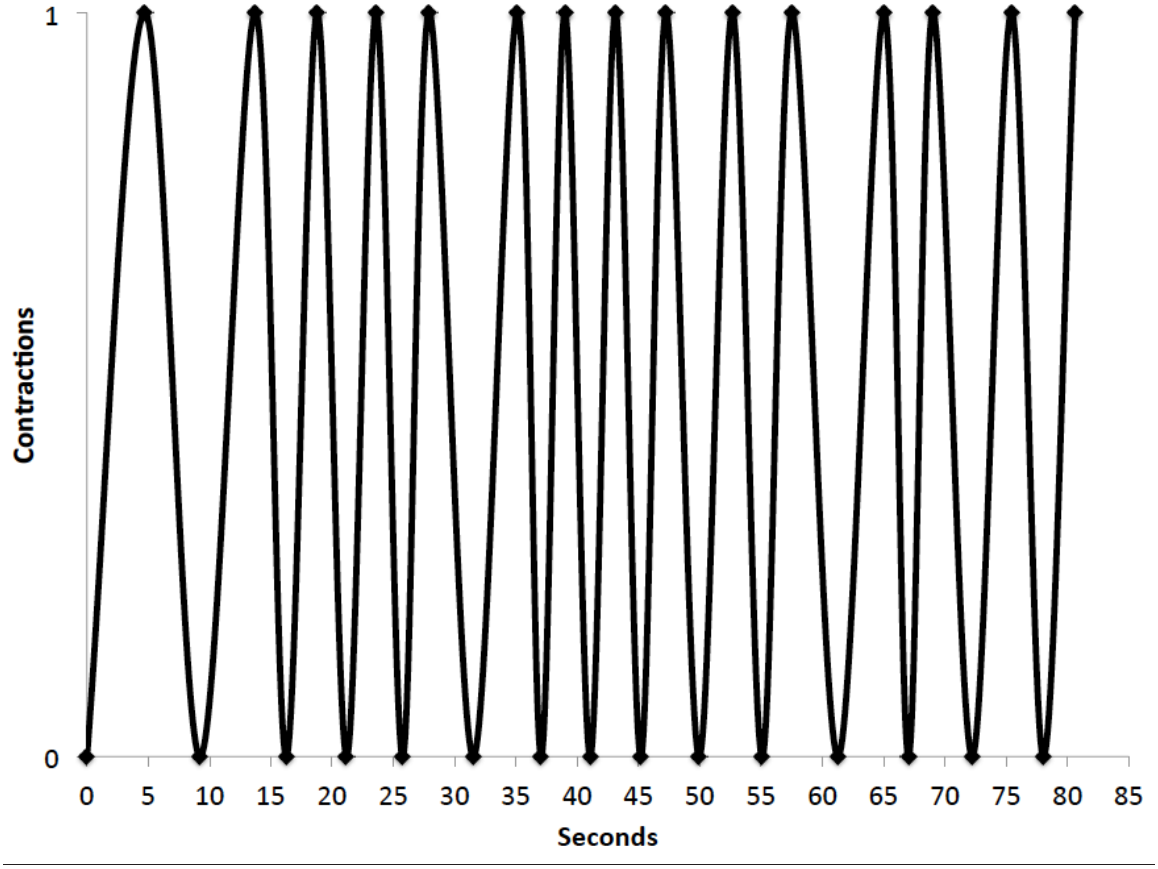


Table 2.1. The affects of neurotransmitters on muscle contractions in *Culex pipiens* seminal vesicles. These results delineate which receptor is being activated. N=3.

Neurotransmitter	Muscle Contractions?	Receptor?
Octopamine	No	OAR (GPCR)
Serotonin	No	5HTR (GPCR)
Acetylcholine	Yes	nAChR or mAChR?
Pilocarpine	No	mAChR (GPCR)
Nicotine	Yes	nAChR (ion gated channel)

Figure 2.5A. Staining of seminal vesicles and accessory glands with α-bungarotoxin. A. Seminal vesicles stained with with α-bungarotoxin-TRITC alone. N=2.

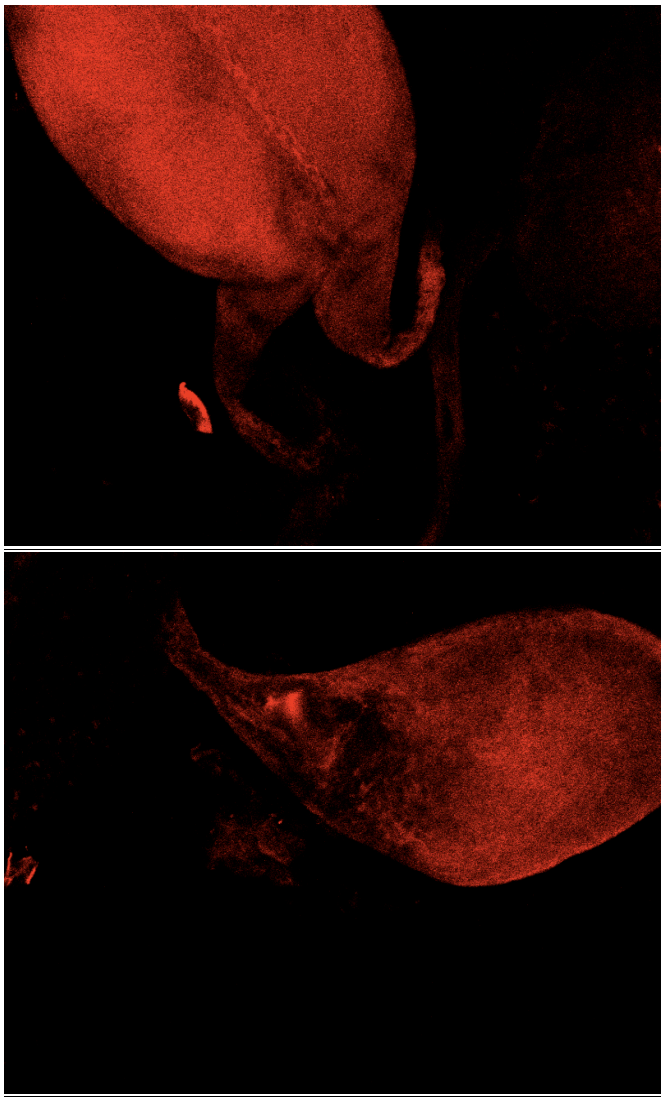


Figure 2.5B. Staining of seminal vesicles and accessory glands with α-bungarotoxin. B. Affect of nicotine pre-treatment on α-bungarotoxin-TRITC staining of seminal vesicles suggesting that an homomeric α7 nicotinic acetylcholine receptor is not responsible for activating muscle contractions in the *Culex* seminal vesicles. N=2.

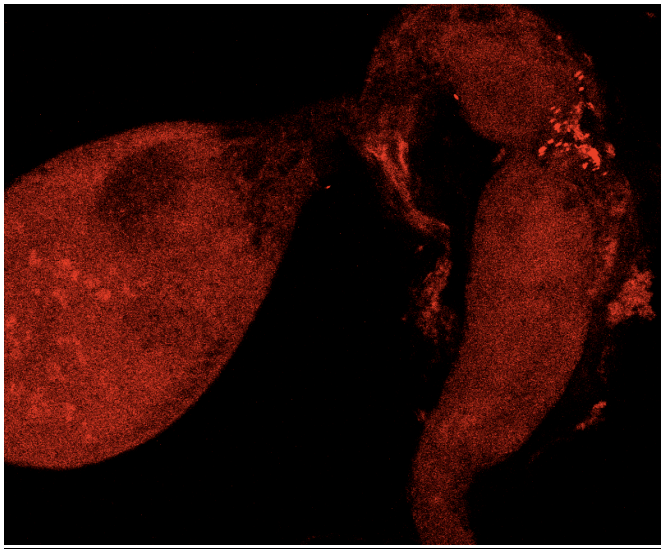


Table 2.2. List of common nicotinic acetylcholine receptor combinations, binding sites, α-bungarotoxin sensitivity, and similarities to subunits found in the *Culex* proteome.

Components/location	**Binding site**	**α-Bungarotoxin sensitive**	**Present in Culex proteome**
Adult: α1 e α1 β1 δ Postsynaptic NMJs	α1/e α1/δ	✓	✗
Fetal: α1 γ α1 β1 δ Extrajunctional	α1/γ α1/δ	✓	✗
(α7)5 CNS and PNS; Developing muscle	α7/α7	✓	EDS44838.1 EDS35062.1 EDS25902.1 XP_001870615.1 XP_001868000.1 XP_001867056.1
α3 β4 α3β4 β4 α3 β2 α3β4 α5 Ganglion	α3/β4 α3/β2	✗	✗
α4 β2 α4β2 β2 Brain	α4/β2	✗	XP_001862560.1 EDS37198.1 EDS42975.1 XP_001866191.1
(α9)5 Cochlea, hair cells	α9/α9	✓	✗

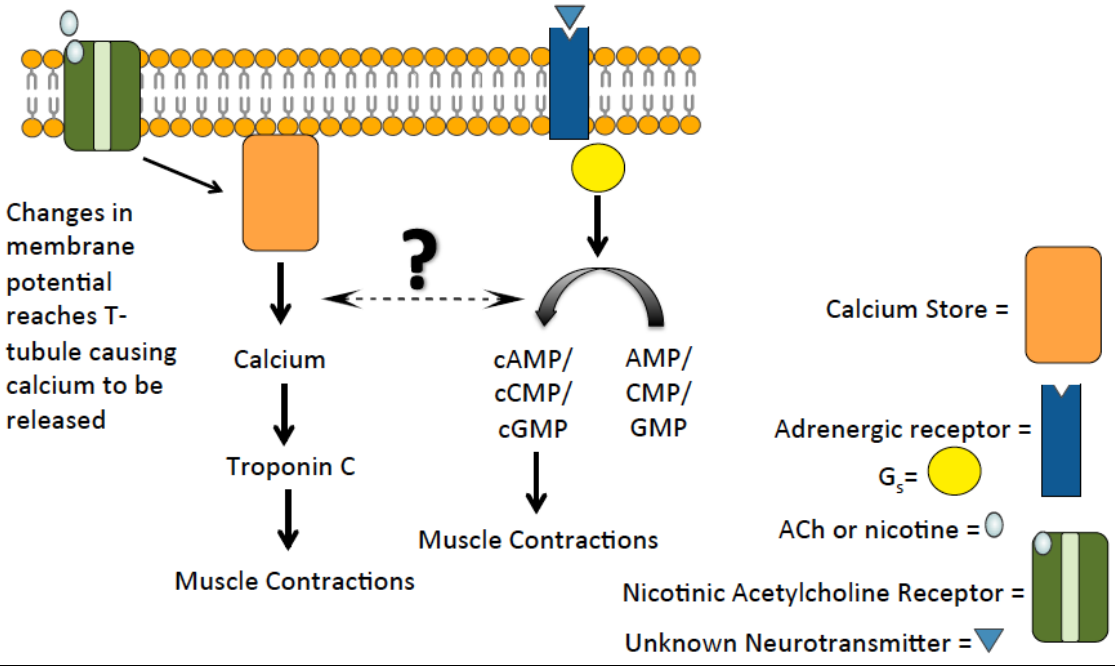
Table 2.3. Affects of pharmacological agents on muscle contractions in *Culex pipiens* seminal vesicles indicating which pathways activate muscle contractions. N=3.

Activator	Muscle Contraction	Pathway Activated	Mode of Action
Caffeine	No	PKA/PKG/PKC	PDE suppression
M-3M3FBS	No	PLC	Activates PLC
IBMX	Yes	PKA/PKG/PKC	PDE suppression

Table 2.4. Affects of pharmacological agents on muscle contractions in *Culex pipiens* seminal vesicles indicating which calcium stores activate muscle contractions. N=3.

Activator	Muscle Contraction	Calcium Store	Mode of Action
Thapsigargin	No	Smooth Endoplasmic Reticulum	SERCA inhibition
Acetylcholine then ryanodine	Inhibits Ach stimulated contractions	Sarcoplasmic endoplasmic reticulum	Inhibits ryanodine receptor
Cyclopiazonic acid	No	Sarcoplasmic endoplasmic reticulum	SERCA inhibition

Figure 2.6. Diagram illustrating the proposed mechanism of muscle contraction activation in male *Culex* seminal vesicles. Acetylcholine activates a cholinergic receptor causing a change in membrane potential, which reaches the T-tubule and causes the release of calcium. Calcium binds to a binding site on troponin thereby exposing the myosin-binding site on actin. Myosin then binds actin and hydrolyzes ATP resulting in a muscle contraction. An alternative pathway that could result in muscle contractions is binding of an unknown neurotransmitter to an adrenergic receptor, which activates a G-protein coupled receptor (GPCR). Actiavtion of the GPCR then leads to the production of cyclic nucleotides, which results in muscle contractions. Clip art was taken Servier Medical Art Reproduction kit (http://www.servier.com/Powerpoint-image-bank) and used to produce a working model.



CHAPTER 3

Characterization of Trypsin-Like Proteases in *Culex* Accessory Glands

Characterization of Trypsin-Like Proteases in *Culex* Accessory Glands

Abstract

In most animals, sperm are stored in a quiescent state in the male reproductive tract and only initiate motility when released into either the female reproductive tract, or in the case of broadcast spawners, into the external environment. Secretions from male accessory glands into the female reproductive tract can provide many important factors for sperm viability and storage and aid in sperm competition. The male accessory glands might also provide a factor that activates sperm motility and, in several insects, a serine protease has been implicated. Our previous studies have shown that, in *Culex quinquefasciatus*, both male accessory gland extracts and trypsin (from porcine pancreas) are sufficient to initiate sperm motility *in vitro*.

The objective of this study was to identify and characterize trypsin-like enzymes produced in the *Culex* male accessory glands. Mass spectrometry was used to analyze accessory gland proteins and this preliminary proteomic analysis identified 3 trypsin-like proteases (trypsin, trypsin4, and trypsin7). To spectrophotometrically characterize the trypsin-like enzymatic activity in soluble extracts of the accessory glands, we used the chromogenic trypsin substrate benzoyl-arginyl ethyl ester (BAEE). Trypsin activity in accessory glands was robust with a pH optimum of 8, similar to vertebrate trypsins, although the *Culex* trypsin was active over a substantially narrower pH range than the vertebrate enzyme. To confirm the presence of specific accessory gland trypsins, the entire contents from *Culex* accessory glands (total protein) were fractionated using a soybean trypsin inhibitor (SBTI) -agarose affinity column and eluted fractions were run

on an SDS PAGE gel and bands identified by silver stain. A small number of bands in the predicted molecular mass range (25-30 kDa) that had affinity with the SBTI were identified. Taken together, these data demonstrate the presence of trypsin-like activity and several trypsin-like proteins in the accessory glands of *Culex*.

Introduction

Mosquitoes are vectors of a variety of diseases that are responsible for more human deaths than any other animal species on earth (WHO, 2004). Mosquitoes from the genus *Culex* are the primary vectors of West Nile Virus in North America and current strategies used to control populations are hampered by the development of insecticide resistance. For example, pyrethroids are widely used for mosquito control but development of pyrethroid resistance has already been reported in several *Culex* species (Corbel et al., 2007; Li and Liu, 2010) as well as other mosquito taxa around the globe (Ransin et al., 2011; Asidi et al., 2012; Wondji et al., 2012). Consequently, it is necessary to understand unique aspects of mosquito biology in order to develop novel population control strategies. Disrupting reproduction, including the activation of sperm motility, could provide a powerful approach to develop novel targeted vector control strategies.

The insect sperm flagellum, as in many animals, is the sole motility apparatus that propels the male gamete through the female reproductive tract to the egg. Following spermatogenesis, mature sperm are stored in a quiescent state and, following copulation, sperm and accessory gland secretions are deposited into the female reproductive tract. The signaling cascade leading to sperm motility activation in some insects, including *Culex* mosquitoes, has only been partially investigated and previous studies have shown that both accessory gland extracts and trypsin are sufficient to activate sperm motility (Thaler et al., 2014). Accessory gland secretions include many components including carbohydrates, proteins, and lipids (Gillott, 2003). In particular, accessory gland proteins span a diverse set of functions, including both proteases and serine protease inhibitors

(Baldini et al., 2012).

Proteolysis is known to regulate several processes that are important for successful reproduction in insects. In *Drosophila*, a seminal metalloprotease-1 that is activated in a stepwise manner in the male reproductive tract and requires female factors for processing and maximal activity is involved in the processing of ovulin, which increases ovulation rates after mating (Avila et al., 2011; Heifetz et al., 2005; Rubenstein and Wolfner, 2013; LaFlamme et al., 2014). In several insect species, proteases directly activate sperm motility (Aigaki et al., 1994; Shepherd, 1974; Shepherd, 1974). In silk moths, initiatorin, a serine protease that is present in the prostatic gland, initiates sperm motility following ejaculation (Aigaki et al., 1994). In other insect species, such as Saturniids, endogenous proteases activate sperm motility (Shepherd, 1974; Shepherd, 1974), while sperm from the water strider, *Aquarius remigis*, are activated by trypsin via a protease-activated receptor 2 (PAR2) pathway that triggers a downstream MAPK signaling cascade (Miyata et al., 2011). More recently, our laboratory has described the activation of *Culex* sperm by a trypsin-like molecule that results in the transition of a weakly oscillating waveform to rapid forward progressive sperm motility in just a few minutes (Thaler et al., 2014). However, to date, the identity of the accessory gland components responsible for this activation of motility are unknown.

In this study, trypsin-like proteases from the *C. pipiens* accessory glands were identified and characterized. We used a proteomics approach to identify five trypsin-like proteases and several protease inhibitors in the accessory glands. These trypsin-like proteases are active in the accessory glands, as assessed by an enzymatic assay, and

several are trypsin-like, as indicated by their retention on a soybean trypsin inhibitor column. Further, these accessory gland trypsins operate over a narrow pH range, compared to mammalian pancreatic enzymes, suggesting that their activity is tightly regulated by factors in the male and/or female reproductive tracts.

Materials and Methods

Materials

Methanol, glycine, BSA, sodium citrate, Na_2HPO_4, NaH_2PO_4, NaCl, $CaCl_2$, Tris-HCl, and Tris-base were purchased from Fisher Scientific (Chino, CA). The Pierce bicinchoninic acid (BCA) protein assay kit was purchased from Thermoscientific (Waltham, MA). Acrylamide and Bromophenol Blue were purchased from Bio-Rad (Hercules, CA). Sodium Dodecyl Sulfate was purchased from CalBiochem. TEMED was purchased from Invitrogen (Waltham, MA). Porcine pancreas trypsin, glycerol, Ammonium Persulfate, β-mercaptoethanol, and N α-Benzoyl-L-arginine ethyl ester (BAEE) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Male *Culex pipiens* were obtained from a colony maintained by Dr. Edward Platzer in the Department of Biology at the University of California, Riverside. One to five male mosquitoes were placed in 1.25 ounce solo cups (product #P125, Solo Cup Company) with lids (product #PL1, Solo Cup Company). Dilute sugar water was added to each cup to humidify the chamber and provide food. Mosquitoes were anesthetized by inserting a small piece of chloroform-soaked cotton into the cup. Subsequently, the male reproductive tract and accessory glands were dissected from the mosquitoes as previously described (Thaler et al., 2014).

Protein concentration determination

Total protein concentrations of accessory gland extracts were determined using a BCA assay (Walker, 2009). Protein was solubilized using sonication and then incubated in BCA solution for two hours and the absorbance measured spectrophotometrically at 562 nm. BSA was used as the standard.

Enzyme Extraction from Whole Accessory Glands

Animals were dissected and their accessory glands were removed and placed in 100 mM Tris-HCl (pH 8.0). Once the dissections were completed, accessory glands were sonicated (Branson Sonifier 250, VWR Scientific) three times for 15 seconds at 40% output and then vortexed briefly. The extract was kept on ice until used for enzymatic assays.

Enzyme Assays

The trypsin-like activity was quantified using the chromogenic substrate BAEE. The reaction mixture consisted of 0.25 mM BAEE diluted in 100 mM Tris-HCl (pH 8.0), and increasing concentrations of enzyme (either crude extract from accessory glands or porcine pancreatic trypsin (control)). The porcine trypsin reaction mixtures were incubated for 3 minutes and the accessory gland extracts were incubated for 7 hours. Absorbance changes from the hydrolyzed chromogenic substrate were measured using a spectrophotometer (DU-640, Beckmann Instruments, Brea, CA; $\lambda = 254$ nm). Background absorbance was determined by measuring the substrate alone over the same

time course, and was subtracted from all assay data. The specifics of each assay are discussed in subsequent sections. All experiments were performed in triplicate.

Enzymatic activity with accessory glands

Accessory gland homogenates were incubated in 0.25 mM BAEE in 100 mM Tris-HCl (pH 8.0). The change in absorbance at 253 nm was measured until the curve reached a plateau. Reaction rates were determined using Beer's Law (molar extinction coefficient of BAEE = 808 M^{-1} cm^{-1} at 254 nm) and the slope of a best-fit line was determined by linear regression. Background absorbance was determined by measuring substrate alone over 7 hours and obtaining the slope. Background was subtracted from all assay data. Assays were repeated three times.

Determination of the optimal pH for enzyme activity

To determine the optimal pH for trypsin-like activity, enzymes were incubated in 0.25 mM BAEE in different buffers for 7 hours: 100 mM sodium citrate buffer for pH <6, 100 mM NaH_2PO_4 buffer at pH 7 and 100 mM Tris–HCl buffer at ≥pH 8. At the end of 7 hours, changes in absorbance were measured using a spectrophotometer (DU-640, Beckman Instruments, Brea, CA; $\lambda = 253$). Subsequently, the effective pH range was determined by normalizing the data to the optimal pH for the porcine pancreas trypsin and the trypsin-like proteins present in the accessory glands. Assays were repeated three times.

Mass spectrometry

Accessory glands were dissected from the male mosquitoes into PBS and subsequently rinsed in fresh PBS. After rinsing with PBS, accessory glands were solubilized using sonication. Accessory gland extracts were sent to the Biomolecular and Proteomics Mass Spectrometry Facility at UC San Diego for mass spectrometry analysis. The *Culex pipiens* genome (available at https://www.vectorbase.org/organisms/culex-quinquefasciatus) was used to identify accessory gland proteins from the mass spectrometry data. The data were subsequently inspected to determine which proteases and protease inhibitors were present in the accessory glands. Mass spectrometry was performed twice. In the first mass spectrometry run 226 proteins were identified and in the second mass spectrometry run 242 proteins were identified. Positive identifications consisted of proteins identified by at least 2 peptides with matches of >95% confidence.

Soybean-Trypsin Inhibitor Agarose Gel

A column of SBTI-agarose (0.5 mL) was equilibrated with 1 mL buffer (TBS, pH 8.0). *Culex* accessory gland extract (200 µL) was applied to the column and subsequently eluted with 75 mM glycine/500 mM NaCl, pH 3.0. One-drop fractions (~50 µL) were collected in microfuge tubes. For the positive and negative control column samples, the pH of each fraction was increased by adding 2 µL of 1 N NaOH. Aliquots (40 µL) of each fraction were boiled in Laemmli sample buffer (62.5 M Tris-HCl (pH 6.8) 10% glycerol, 2% SDS, 5% β-mercaptoethanol, trace bromophenol blue) for 5 min, and the samples were then separated by SDS-PAGE using a 12% separating gel. For the accessory gland samples, the flow through (FT) fractions and the eluate (EL) fractions

were each pooled and concentrated using Vivaspin filters (NMWCO, 10 kDa). Phenylmethylsulfonyl flouride (PMSF, final concentration 1 mM) was added to the pooled FT and EL prior to concentrating in order to block degradation of the samples by any endogenous proteases. Molecular weight standards (Precision Plus Protein Kaleidoscope Standards, Bio-Rad, Hercules, CA) were included on the gel. Positions and sizes of the markers are indicated to the left of the gels (Figure 4). Gels were stained with silver nitrate (Merrill and Pratt, 1986). Subsequently, gels were imaged using the Bio-Rad Gel Doc™ EZ System with the silver stain settings. Following optimization of column conditions, two independent trials were conducted for both the control and accessory gland samples.

Results

Mass Spectrometry

After accessory gland samples were collected and analyzed against the *Culex pipiens* genome, mass spectrometry data was examined to determine specific proteases that were present in the accessory glands. Fourteen proteases were identified in the accessory glands, including three predicted trypsins and a serine protease, ranging in size from 26 kDa – 69 kDa (Table 1). Serine proteases represent potential activators of sperm motility, since previous work showed that either accessory gland extracts, or purified bovine pancreatic trypsin, are capable of activating sperm motility in vitro (Thaler et al., 2014). In addition, five serine protease inhibitors were also identified in the accessory glands (Table 1), suggesting that they may play a role in downregulating protease activity until mixing with sperm during ejaculation.

Enzymatic activity in the accessory glands

Trypsin-like proteins catalyze hydrolysis of internal peptide bonds on the carboxyl side of arginine and lysine residues (Schwert et al., 1948). Trypsin-like activity from *C. pipiens* accessory glands was characterized using a chromogenic substrate, N_a-Benzoyl-L-arginine-ethyl-ester-hydrochloride (BAEE). Absorbance at 253 nm increased linearly with increasing concentrations of either a porcine pancreatic trypsin (Figure 1A) or the whole protein extract from accessory glands (Figure 1B), indicating that trypsin-like activity is detectable in accessory gland extracts.

pH optimum

Similar to porcine pancreatic trypsin, *C. pipiens* accessory gland trypsin-like protease had an optimal pH of 8.0 when BAEE was used as the substrate (Figure 2). However, porcine pancreatic trypsin was active over a broad pH range (~ pH 5 – pH 10), whereas accessory gland trypsins exhibited a much narrower range of activity with detectable activity from ~pH 6 – pH 9 (Figure 3).

Soybean Trypsin Inhibitor (SBTI) Agarose Affinity Chromatography

We attempted to purify active trypsin molecules from accessory glands using an SBTI-agarose affinity column. An SBTI-agarose affinity column is a type of affinity chromatography, a biochemical method to separate a heterogenous solution based on enzyme-inhibitor interaction. Briefly, soybean trypsin inhibitor is conjugated to agarose beads and loaded into a column. A solution containing trypsin can then be run across the beads with SBTI conjugatedand the SBTI will only interact with trypsin. The trypsin can then be released from the SBTI by exposing it to high pH or high ionic strength. To calibrate the column, we mixed BSA and purified porcine pancreatic trypsin and added it to the column. As expected, BSA did not bind to the column, and while pancreatic trypsin bound with sufficient affinity until the buffer was switched to high ionic strength/low pH conditions. As previously stated the retained protein has a molecular mass of ~23 kDa (Figure 4A). Once calibrated, the *Culex* accessory gland homogenate was fractionated using the same conditions as the control. The flow-through (FT) fraction contained a number of proteins that did not bind the SBTI-agarose, while the eluted fraction (EL)

contained a number of unique proteins that differed from the FT. In particular, three accessory gland polypeptides with molecular masses of 21 kDa, 23 kDa, and 26 kDa, consistent with the predicted masses of trypsins identified in the mass spectrometry results (Figure 4B). Several other trypsin-like proteases that are found in the mass spectrometry data apparently were too low in abundance to detect.

Discussion

In many eukaryotic organisms, sperm flagellum propels sperm through the female reproductive tract. However, sperm are maintained in a quiescent state in the male reproductive tract until they come in contact with specific chemical signals. The mechanism of sperm activation has only been studied in a few species, despite being a nearly ubiquitous process in both protostome and deuterostome lineages.

Sperm motility activation in many animals is regulated by extracellular cues such as changes in viscosity or pH (Yoshida et al., 2008). Ascidians control motility activation using a sulfated steroid that the egg secretes (Izumi et al., 1999; Yoshida et al., 2002; Matsumoto et al., 2013); whereas fish and amphibians control motility activation via changes in intracellular pH, calcium or cAMP (Cherr et al., 2008; Ingermann et al., 2008; Zilli et al., 2008; Hu et al., 2009; Zilli et al., 2011; Takei et al., 2012; Ohta et al., 2010; Watanabe et al., 2010; Tholl et al., 2009; Watanabe et al., 2011; Takayama et al., 2012). In some organisms, chemical factors that activate sperm motility are released by the egg and, therefore, represent the first communication between sperm and egg.

In many insect taxa, initiation of motility is activated by a serine protease such as trypsin or tryptase (Osanai and Baccetti, 1993; Friedländer et al., 2001; Miyata et al., 2012; Thaler et al., 2014). In *Culex*, sperm stored in seminal vesicles initiate sperm motility only after they are exposed to either trypsin or an endogenous initiator present in the accessory glands (Thaler et al., 2014). In this study we identified a number of serine proteases and several trypsins that are present within the accessory glands. Given our previous results (Thaler et al., 2014), these accessory gland serine proteases are likely

involved in activating *Culex* sperm motility during mixing of accessory gland fluids with sperm during ejaculation. It is possible that serine protease inhibitors that are present in the accessory gland proteome are involved in regulating activation of sperm motility in order to ensure that the sperm remains quiescent until transferred or ready to be transferred to the female reproductive tract. This level of regulation would be necessary to maintain sperm quiescence and minimize energy utilization prior to entering the female reproductive tract. Other arthropods have serine proteases and serine protease inhibitors in their accessory glands (Baldini et al., 2012; Takemori and Yamamoto, 2009; Walker et al., 2006; Sonenshine et al., 2011), indicating that this mechanism of activation may be more broadly conserved across this phylum. Alternatively, analogous to *Drosophila* (LaFlamme et al., 2014), these serine proteases and serine protease inhibitors may play multiple reproductive roles in both males and females.

In addition to regulation by protease inhibitors, pH may play a critical role in either male or female reproductive tracts in controlling activation of accessory gland trypsins. In this study, we showed that although the pH optimum was virtually identical for porcine pancreatic and *Culex* accessory gland trypsins, the range of activity was substantially narrower for the accessory gland trypsin. Another insect species, *Rhyzopertha dominica,* has trypsin like activity in the midgut with a pH range between pH 6.5 - pH 9.5 (Zhu and Baker, 1999). Mammalian pancreatic proteolytic activity must accommodate a wider pH range because of the emptying of acidic contents from the stomach into the duodenum that is buffered by bicarbonate, but the narrower pH range of the accessory gland trypsin likely has implications for its function in reproduction. It is

possible that the reproductive tract of a receptive and fertile female maintains a narrow pH range of ~8 within a specific region of the reproductive tract to ensure that sperm activation occurs at the optimal time and place to result in successful fertilization. This mechanism would be especially important if the accessory gland fluid and sperm are transferred simultaneously into the female reproductive tract.

In both *Culex* and *A. remigis*, trypsin activation of sperm induces a MAPK-pathway (Thaler et al., 2014) resulting in flagellar motion. Specifically, we have proposed a model whereby trypsin cleavage activates a Protease Activated Receptor 2 (PAR2) -like protein in order to activate sperm motility (Miyata et al., 2012; Thaler et al., 2014). PARs are GPCRS that are activated by the cleavage of a canonical sequence by a specific protease (Cottrell et al., 2003). Trypsin cleaves PAR2 between the arginine and serine residues of an RSLIGKV (human) or an RSLIGRL (mouse) sequence (Cottrell et al., 2003) to activate the receptor and stimulate MAPK signaling (Cottrell et al., 2003; Ramachandran et al., 2009). Currently, the only PAR2 known to exist in insects is present in the *A. remigis* sperm flagellum as shown via an anti-PAR2 antibody directed at the N-terminus of the mouse PAR2 (Thaler et al., 2014), providing further evidence that trypsin cleaves a PAR2-like protein in order to activate sperm motility. This particular method of sperm motility activation is, to date, unique to insects. The mechanism for motility activation in other organisms does not depend on trypsin cleavage. In mammals, sperm motility is acquired by the activation of an adenylyl cyclase (SACY) by bicarbonate (HCO_3^-), which activates cAMP signaling and downstream protein tyrosine phosphorylation (Chen et al., 2000; Visconti et al., 1995).

Since reproduction is necessary to maintain populations, targeting reproductive pathways can help to reduce pest populations, including mosquitoes. We have demonstrated the presence of trypsin and trypsin-like molecules in the accessory glands of *Culex* that are likely responsible for activating sperm motility. In the future, knockout mosquitoes can be produced targeting the trypsin and trypsin-like molecules that have been identified in the *Culex* accessory gland proteome. Alternatively, further studies will determine the specific target of trypsin, and knockout mosquitoes can be produced targeting the trypsin substrate. By targeting either the trypsin-like enzyme or the conjugate substrate with gene knockout technology, mosquito reproduction will be effectively controlled, resulting in the attenuation of many vector-borne diseases.

References

Aigaki T., Kasuga H., Nagaoka S. and Osanai M. (1994). Purification and partial amino acid sequence of initiatorin, a prostatic endopeptidase of the silkworm, *Bombyx mori. Insect Biochem. Mol. Biol.* 24: 969–975.

Asidi A., N'Guessan R., Curtis C. and Rowland M. (2012). Loss of household protection from use of insecticide-treated netsagainst pyrethroid-resistant mosquitoes, *Benin. Emerg. Infect. Dis.*18: 1101–1106.

Avila F. W., Sirot L. K., LaFlamme B. A., Rubinstein C. D. and Wolfner M. F. (2011). Insect seminal fluid proteins: identification and function. *Annu Rev Entomol* 56: 21-40.

Baldini F., Gabrieli P., Rogers D.W. and Catteruccia F. (2012). Function and composition of male accessory gland secretions in *Anopheles gambiae*: A comparison with other insect vectors of infectious diseases. *Pathog. Glob. Health.* 106(2): 82–93

Chen Y., Cann M.J., Litvin T.N., Iourgenko V., Sinclair M.L., Levin L.R. and Buck J. (2000): Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science.* 289: 625-8.

Corbel V., N'Guessan R., Brengues F., Chandre F., Djogbenou L., Martin T., Akogbeto M., Hougard J.M. and Rowland M. (2007). Multiple insecticide resistance mechanisms in Anopheles gambiae and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica.* 101: 207–216.

Cottrell G. S., Amadesi S., Schmidlin F. and Bunnett N. (2003) Protease-activated receptor 2: activation, signalling and function. *Biochem. Soc. Trans*. **31**: 1191–1197

Cherr G.N., Morisawa M., Vines C.A., Yoshida K., Smith E.H., Matsubara T., Pillai M.C., Griffin F.J. and Yanagimachi R. (2008). Two egg-derived molecules in sperm motility initiation and fertilization in the Pacific herring (Clupea pallasi). *Int J Dev Biol* 2008; 52:743–752.

Friedländer M., Jeshtadi A. and Reynolds S.E. (2001): The structural mechanism of trypsin-induced intrinsic motility in Manduca sexta spermatozoa in vitro. *J Insect Physiol.* 47, 245-55.

Gillott C. (2003). Male accessory gland secretions: modulators of female reproductive physiology and behaviour. *Ann. Rev. Entom.* 48:163–84.

Heifetz Y., Vandenberg L. N., Cohn H. I. and Wolfner M. F. (2005). Two cleavage products of the *Drosophila* accessory gland protein ovulin can independently induce ovulation. *Proc Natl Acad Sci U S A* 102: 743-748

Hu J., Zhang Y., Zhou R. and Zhang Y. (2009). Changes in extracellular osmolality initiate sperm motility in freshwater teleost rosy barb *Puntius conchonius*. *Theriogenology* 72:704–710.

Ingermann R.L., Holcomb M., Zuccarelli M.D., Kanuga M.K. and Cloud J.G. (2008). Initiation of motility by steelhead (*Oncorhynchus mykiss*) sperm: membrane ion exchangers and pH sensitivity. *Comp Biochem Physiol A Mol Integr Physiol* 151:651–656.

Izumi H., Marian T., Inaba K., Oka Y. and Morisawa M. (1999) Membrane hyperpolarization by sperm-activating and -attracting factor increases cAMP level and activates sperm motility in the ascidian *Ciona intestinalis. Dev Biol* 213:246–256.

Laflamme B.A., Avila F.W., Michalski K. and Wolfner M.F. (2014). A *Drosophila* protease cascade member, seminal metalloprotease-1, is activated stepwise by male factors and requires female factors for full activity. *Genetics.* 196(4): 1117-29.

Li T. and Liu N. (2010). Inheritance of permethrin resistance in *Culex quinquefasciatus*. *J. Med. Entomol*. 47: 1127–1134.

Matsumori N., Hiradate Y., Shibata H., Oishi T., Shimma S., Toyoda M., Hayashi F., Yoshida M., Murata M. and Morisawa M. (2013). A novel sperm-activating and attracting factor from the ascidian *Ascidia sydneiensis. Org Lett*. 15:294–297.

Merril C.R. and Pratt M.E. (1986) A silver stain for the rapid quantitative detection of proteins or nucleic acids on membranes or thin layer plates, *Anal Biochem* 156, 96110

Miyata H., Thaler C., Haimo L. and Cardullo R. (2011). Protease Activation and the Signal Transduction Pathway Regulating Motility in Sperm from the Water Strider *Aquarius remigis. Cytoskeleton*, 69:207–220

Ohta M., Kubo H., Nakauchi Y. and Watanabe A. (2010). Sperm motility-initiating activity in the egg jelly of the externally-fertilizing urodele amphibian, *Hynobius lichenatus. Zoolog Sci* 27:875–879.

Osanai M. and Baccetti B. (1993): Two-step acquisition of motility by insect spermatozoa. *Experientia*. 49: 593-5.

Ramachandran R., Mihara K., Mathur M., Rochdi M.D., Bouvier M., Defea K. and Hollenberg M.D. (2009). Agonist-biased signaling via proteinase activated receptor-2: differential activation of calcium and mitogen-activated protein kinase pathways. *Mol Pharmacol* 76:791–801

Ranson H., N'guessan R., Lines J., Moiroux N., Nkuni Z. and Corbel V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol.* 27: 91–98.

Rubinstein C. D. and Wolfner M. F. (2013). *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proc Natl Acad Sci U S A*.

Schwert G.W., Neurath H., Kaufman S. and Snoke JE. (1948). The specific esterase activity of trypsin. *J Biol Chem*. 172: 221-239

Shepherd JG. (1974). Activation of saturniid moth sperm by a secretion of the male reproductive tract. *J. Insect Physiol.* 20:2107–2122.

Sonenshine D.E., Bissinger B.W., Egekwu N., Donohue K.V., Khalil S.M. and Roe R.M. (2011). First transcriptome of the testis-vas deferens-male accessory gland and proteome of the spermatophore from *Dermacentor variabilis* (Acari: Ixodidae). *PLoS One*. 6(9): e24711.

Takayama-Watanabe E., Campanella C., Kubo H. and Watanabe A. (2012). Sperm motility initiation by egg jelly of the anuran, *Discoglossus pictus*, may be mediated by sperm motility-initiating substance of the internally-fertilizing newt, *Cynops pyrrhogaster. Zygote* 20:417–422.

Takei G.L., Mukai C. and Okuno M. (2012). Transient Ca2. mobilization caused by osmotic shock initiates salmonid fish sperm motility. *J Exp Biol* 215:630–641.

Takemori N. and Yamamoto M.T. (2009). Proteome mapping of the *Drosophila melanogaster* male reproductive system. *Proteomics* 9: 2484–2493.

Thaler C., Miyata H., Haimo L. and Cardullo R. (2014). Waveform Generation Is Controlled by Phosphorylation and Swimming Direction Is Controlled by Ca^{2+} in Sperm from the Mosquito *Culex quinquefasciatus. Biol. Reprod.* 89(6): 1-11.

Tholl N., Naqvi S., McLaughlin E., Boyles S., Bieber A.L. and Chandler D.E. (2011). Swimming of *Xenopus laevis* sperm exhibits multiple gears and its duration is extended by egg jelly constituents. *Biol Bull* 220: 174–185.

Visconti P.E., Bailey J.L., Moore G.D., Pan D., Olds-Clarke P. and Kopf G.S. (1995). Capacitation of mouse spermatozoa I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development*. 121: 1129-37.

Walker M.J., Rylett C.M., Keen J.N., Audsley N., Sajid M., Shirras A.D. and Isaac R.E. (2006). Proteomic identification of *Drosophila melanogaster* male accessory gland proteins, including a pro-cathepsin and a soluble gamma-glutamyl transpeptidase.

Proteome Sci. 4: 9.

Walker J. M. (2009). The bicinchoninic acid (BCA) assay for protein quantitation. *The Protein Protocols Handbook*, 11-15.

Watanabe T., Kubo H., Takeshima S., Nakagawa M., Ohta M., Kamimura S., Takayama-Watanabe E., Watanabe A. and Onitake K. (2010). Identification of the sperm motility-initiating substance in the newt, *Cynops pyrrhogaster*, and its possible relationship with the acrosome reaction during internal fertilization. Int J Dev Biol. 54:591–597.

Watanabe A., Takayama-Watanabe E., Vines C.A. and Cherr G.N. (2011). Sperm motility-initiating substance in newt egg-jelly induces differential initiation of sperm motility based on sperm intracellular calcium levels. *Dev Growth Differ* 53:9–17.

WHO. *The World Health Report 2004: Changing History* (WHO, 2004).

Wondji C. S., Coleman M., Kleinschmidt I., Mzilahowa T., Irving H. and Ndula M. (2012). Impact of pyrethroid resistance on operational malaria control in Malawi. *Proc. Natl. Acad. Sci. USA.* 109: 19063–19070.

Yoshida M., Murata M., Inaba K. and Morisawa M. A. (2002). chemoattractant for ascidian spermatozoa is a sulfated steroid. *Proc Natl Acad Sci USA*. 99:14831–14836.

Yoshida M., Kawano N. and Yoshida K. (2008). Control of sperm motility and fertility: diverse factors and common mechanisms. *Cell. Mol. Life Sci*. 65, 3446-3457

Zhu Y.C. and Baker J.E. (1999). Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochem. Mol. Biol.* 29: 1053–1063

Zilli L., Beirao J., Schiavone R., Herraez M.P., Cabrita E., Storelli C. and Vilella S. (2011). Aquaporin inhibition changes protein phosphorylation pattern following sperm motility activation in fish. *Theriogenology*. 76:737–744.

Zilli L., Schiavone R., Storelli C. and Vilella S. (2008). Molecular mechanisms determining sperm motility initiation in two sparids (*Sparus aurata* and *Lithognathus mormyrus*). *Biol Reprod.* 79:356–366.

Figures and Tables

Table 3.1. Proteases Identified by Mass Spectrometry of *Culex pipiens* Male Accessory Glands. N=2.

Gene ID	Gene name	Predicted Mass (kDa)
CpipJ_CPIJ019596	Serine protease	69
CpipJ_CPIJ017964	Trypsin 7	26
CpipJ_CPIJ007078		27
CpipJ_CPIJ006471	Cathepsin I	38
CpipJ_CPIJ000574	Cathepsin L	36
CpipJ_CPIJ004642	Trypsin	32
CpipJ_CPIJ006867	Transmembrane protease	32
CpipJ_CPIJ007077	Trypsin 4	31
CpipJ_CPIJ014660	Protease m1 zinc metalloprotease	105
CpipJ_CPIJ008439	puromycin-sensitive aminopeptidase	98
CpipJ_CPIJ014907	xaa-pro dipeptidase	53
CpipJ_CPIJ019883	Chymotrypsinogen 2	32
CpipJ_CPIJ017185	Lysosomal aspartic protease	42
CpipJ_CPIJ012757	Neuroendocrine convertase	57

Table 3.2. Serine Protease Inhibitors Identified by Mass Spectrometry of *Culex pipiens* Male Accessory Glands. N=2.

Gene ID	Gene name	Predicted Mass (kDa)
CpipJ_CPIJ014719	Alaserpin	42
CpipJ_CPIJ005227	Serine protease inhibitor	24
CpipJ_CPIJ003759		56
CpipJ_CPIJ006758		44
CpipJ_CPIJ011775	Serpin B6	49
CpipJ_CPIJ015496		20
CpipJ_CPIJ017784	Serpin B8	29
CpipJ_CPIJ010521	dipetalogastin	31
CpipJ_CPIJ010990	Pacifastin	47

Figure 3.1A. Characterization of Enzymatic Activity Using a Chromogenic Substrate. **(A)** Tryptic hydrolysis of 0.25 mM BAEE using increasing concentrations of a control enzyme, porcine pancreatic trypsin. Linear regression: $y=443.3x+86.17$, $R^2= 0.99$. N=3. Error bars show standard error. N=3.

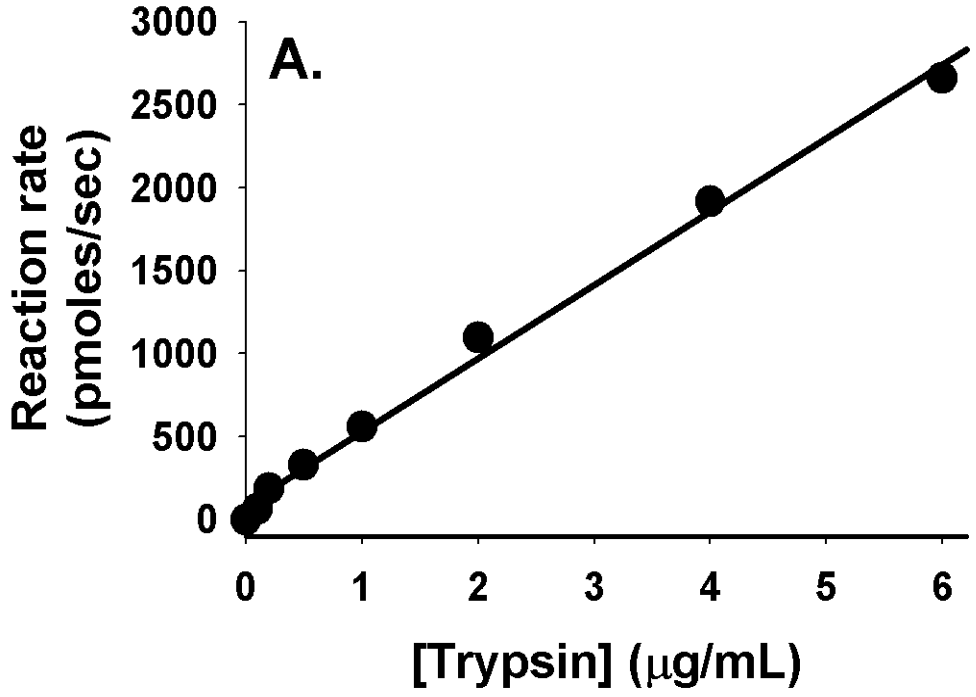


Figure 3.1B. Characterization of Enzymatic Activity Using a chromogenic Substrate. **(B).** Tryptic hydrolysis of 0.25 mM BAEE using increasing concentrations of total protein from *Culex* accessory glands. Linear regression: y=0.69x+1.12, R^2= 0.93. N=3, Error bars show standard error. N=3.

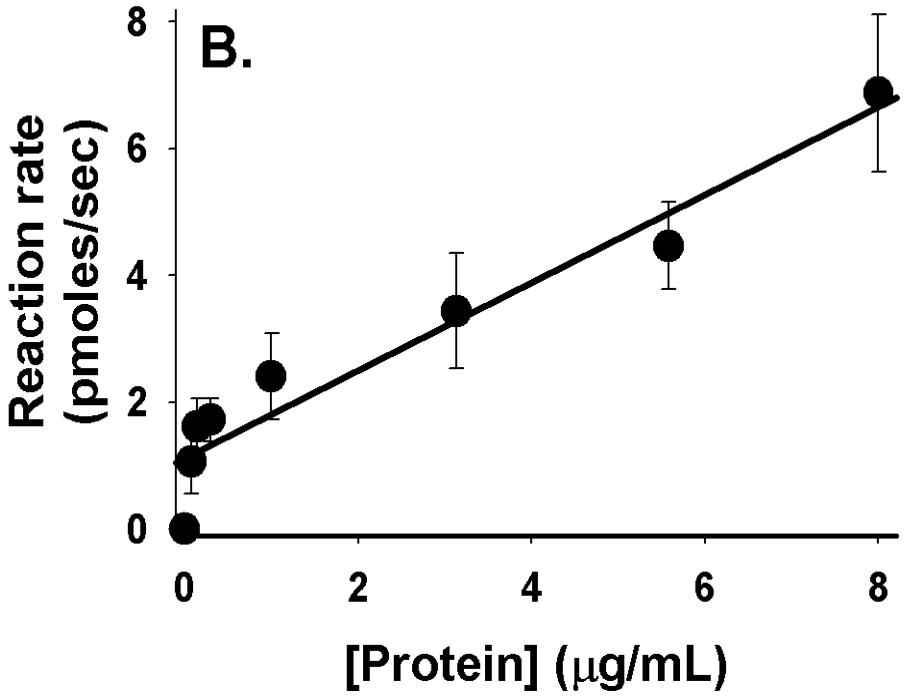


Figure 3.2. The pH dependence of Accessory Gland Trypsins. Tryptic hydrolysis of 0.25 mM BAEE using a range of pH conditions revealed differences in pH sensitivity between the control porcine pancreatic trypsin (open circles) and trypsins in the total protein extract of *Culex* accessory glands (closed circles). N=2.

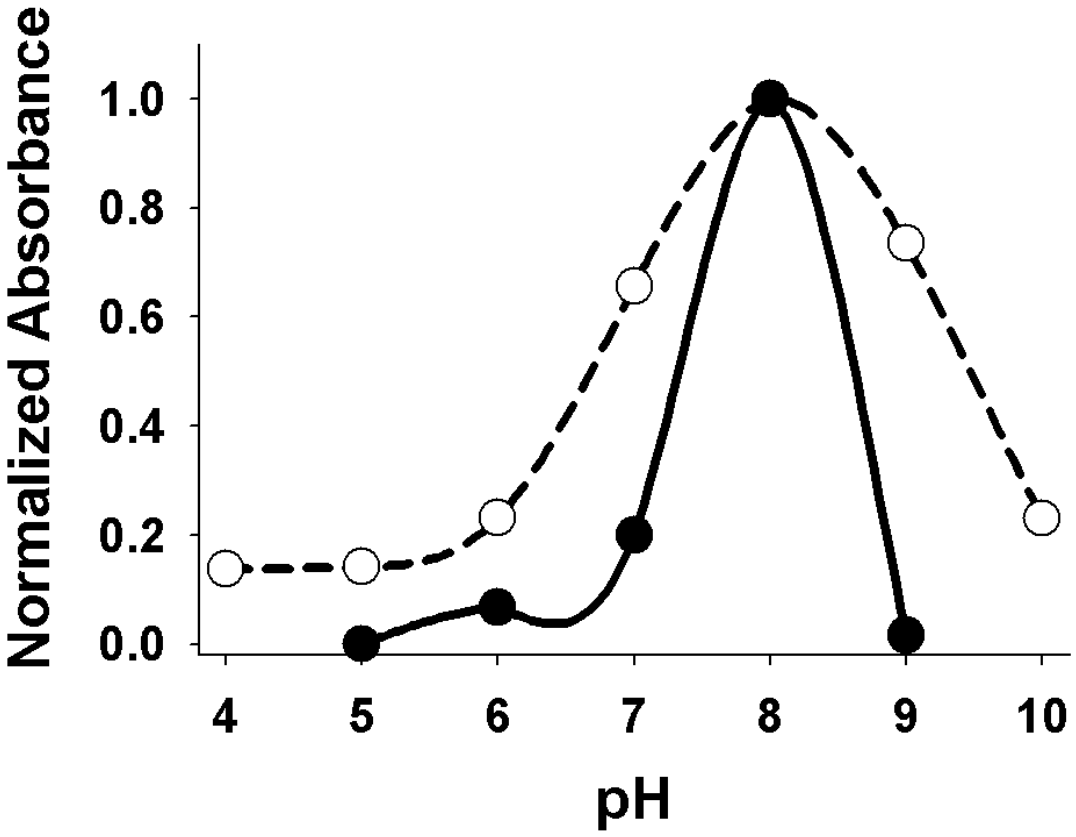


Figure 3.3A. *Culex* Accessory Gland Proteins Bind SBTI. **(A)** The ability of a soybean trypsin inhibitor (SBTI-agarose) column to specifically bind trypsin-like proteins was determined using BSA and porcine pancreatic trypsin as negative and positive controls, respectively. BSA was detected exclusively in the flow through (FT) fractions (lane 1). Trypsin was eluted from the column using a high salt/low pH buffer. Trypsin was first detected weakly in fraction 4, and then more strongly in subsequent fractions (5-8 shown). The positions and sizes of the markers are indicated to the left of the gels. N=2.

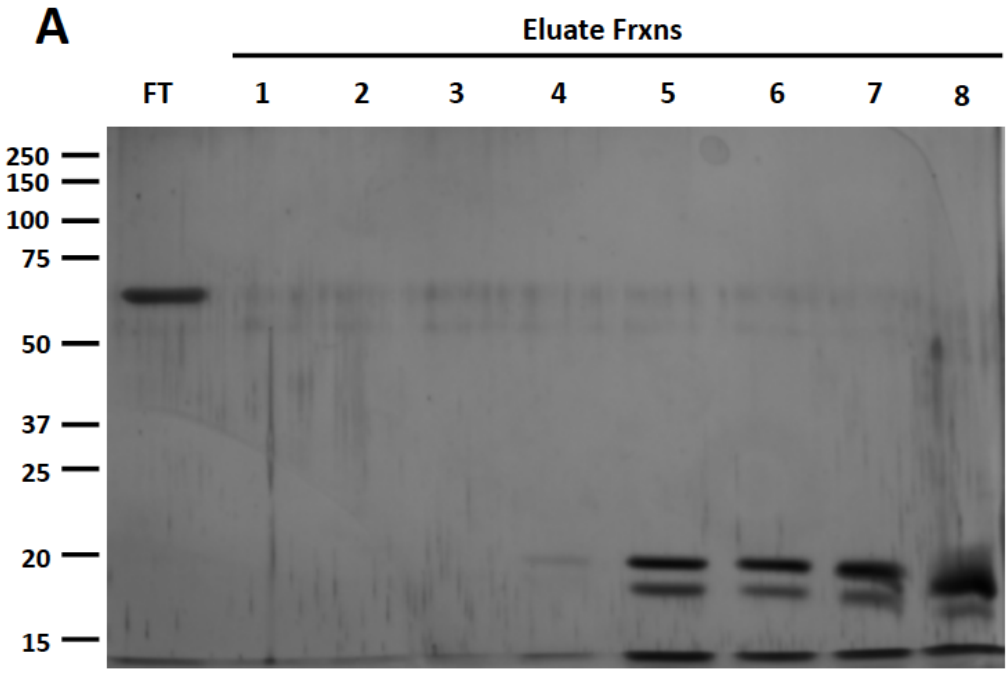
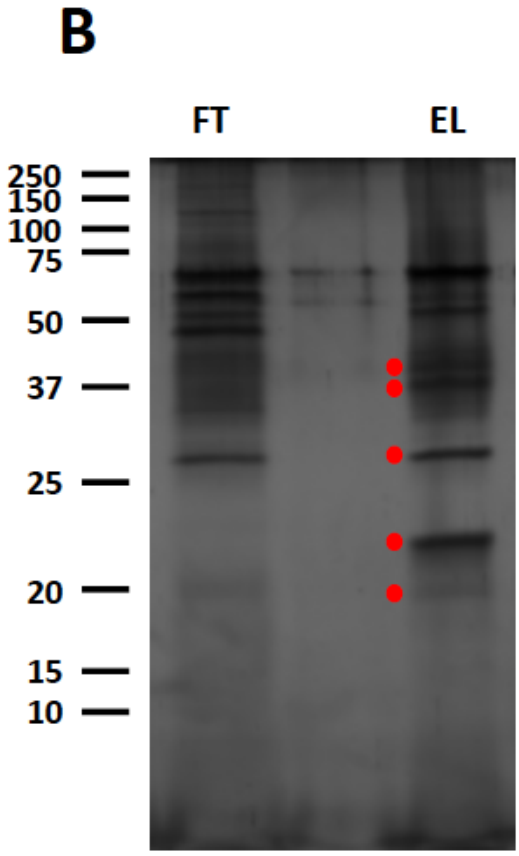


Figure 3.3B. *Culex* Accessory Gland Proteins Bind SBTI. **(B)** When a homogenate of *Culex* accessory glands was fractionated on a SBTI-agarose column, using the same conditions as for the controls, a complex array of proteins was detected in the flow through (FT). Several proteins were enriched or detected exclusively in the eluate (EL, red dots). Proteins that were found not only in the eluate, but also in the flow through may have interacted non-specifically with the column due to their relative abundance in the sample. Three proteins in the eluate have molecular sizes consistent with trypsin-like proteins detected in the MS analysis of the accessory glands (21, 23, and 26 kDa). The positions and sizes of the markers are indicated to the left of the gel lane.



CHAPTER 4

Characterization of Plasma Membrane Associated Type II α-D-mannosidase and β-N-acetylglucosaminidase of *Aquarius remigis* Sperm.

Characterization of plasma membrane associated type II α-D-mannosidase and β-N-acetylglucosaminidase of *Aquarius remigis* sperm.

Abstract

For successful fertilization to occur, molecules on the surface of male and female gametes must recognize each other in a complementary manner. In some organisms, sperm possess a glycosidase on the plasma membrane overlying the head while eggs have glycoproteins that are recognized by those glycosidases resulting in sperm-egg recognition. In this study, two glycosidases, mannosidase and β-N-acetylglucosaminidase, were identified and biochemically characterized in *Aquarius remigis* sperm. The mannosidase has a K_m of 2.36 ± 0.19 mM, a Vmax of 27.49 ± 0.88 pmol/min and a Hill coefficient of 0.94 ± 0.18 at its optimal pH of 7.0. The mannosidase is extracted most efficiently with CHAPSO but is also efficiently extracted with sodium chloride. Mannosidase activity is effectively inhibited by swainsonine, but not by kifunesine, and is significantly reduced in the presence of Mn(2+) and Mg(2+), but not Zn(2+). N-acetylglucosaminidase had a K_m of 0.093 ± 0.01 mM, a Vmax of 153.80 ± 2.97 pmol/min and a Hill coefficient of 0.96 ± 0.63 at its optimal pH of 7.0. N-acetylglucosaminidase was extracted most efficiently with potassium iodide but was also efficiently extracted with Triton X-100 and Zn(2+), but not Ca(2+), Co(2+), Mn(2+) or Mg(2+), significantly inhibited its activity. Taken together, these results indicate that the *A. remigis* sperm surface contains at least two glycosidases that may recognize complementary glycoconjugates on the surface of water strider eggs.

Introduction

Complementary ligand–receptor pairs between gametes play important roles in intercellular signaling, adhesion, and physiological transformations that are necessary for successful fertilization in animals. Carbohydrates appear to be particularly important for these events in both vertebrate and invertebrate systems that have been studied to date and carbohydrates have been suggested to be important in binding and recognition during fertilization. Specifically, glycans may play roles in maintaining sperm in storage or binding to the egg surface (Demott et al., 1995; Dobrinski et al., 1996; Green et al., 2001 Wagner et al., 2002; Tulsiani et al., 1997; Martinez et al., 2000; Miranda et al., 2000 Mengerink and Vacquier, 2001; Rodeheffer and Shur, 2002; Koyanagi and Honegger, 2003; Claw and Swanson, 2012; Chiu et al., 2008; Pang et al., 2011). Thus, glycosidases could be involved in maintaining sperm in storage reservoirs. In mammals, sperm are stored in the oviduct by binding the epithelial surface (Harper, 1973; Hunter, 1981 Hunter and Nichol, 1983; Overstreet and Cooper, 1978; Suarez, 1987; Wilmut and Hunter, 1984; Yanagimachi and Chang, 1963) and oligosaccharides competitively inhibit sperm-epithelium binding in hamster (DeMott et al., 1995), horse (Dobrinski et al., 1996), and pig (Green et al., 2001; Wagner et al., 2002) suggesting that carbohydrates mediate sperm-epithelium binding.

Despite evidence that carbohydrates mediate sperm-egg recognition, the involvement of glycosidases as recognition molecules remains controversial. Additionally, both sperm surface lectin-like molecules and conjugate sugars on the egg surface have been proposed to mediate the gamete recognition step (Tulsiani et al., 1997;

Martinez et al., 2000; Miranda et al., 2000; Mengerink and Vacquier, 2001; Rodeheffer and Shur, 2002; Koyanagi and Honegger, 2003; Claw and Swanson, 2012; Chiu et al., 2008; Pang et al., 2011). Some of these molecules interact through a non-catalytic complex between sperm surface glycosidases or glycosyltransferases and complementary carbohydrates on the extracellular matrices surrounding the egg plasma membrane (Rodeheffer and Shur, 2002). As intracellular enzymes, glycosidases catalyze the hydrolysis of glycosidic linkages within lysosomes, but on cell surfaces their enzymatic cycle is inactivated leading to the formation of a receptor–ligand pair. Enzymes represent an important potential class of receptors since they exhibit a high degree of absolute, group, linkage or stereochemical specificity with their substrate (Seager and Slabaugh, 2010).

Several sperm surface glycosidases have been identified as putative egg surface (e.g., chorion, zona pellucida, etc.) recognition molecules. Examples of glycosidases present on the sperm plasma membrane include mannosidases in mammalian sperm (Tulsiani et al., 1989; Tulsiani et al., 1990; Tulsiani et al., 1995), fucosidases and N-acetylglucosaminidases in tunicates (Godknecht and Honegger, 1991; Matsumoto et al., 2002; Downey and Lambert, 1994), and mannosidases, fucosidases and N-acetylglucosaminidases in flies (Perotti, 2001; Pasini et al., 1999; Intra et al., 2006; Intra et al., 2011; Cattaneo et al., 1997). In mammals, sperm from rats and mice possess a sperm surface mannosidase and the zona pellucida (ZP) surrounding the egg plasma membrane is heavily mannosylated (Tulsiani et al., 1997). In the ascidians *Phallusia mammillata* (Hoshi et al., 1985; Hoshi, 1986; Godknecht and Honegger,

1991; Godknecht and Honegger, 1995) and *Ascidia* sp. (Lambert, 1989), the sperm plasma membrane contains a β-N-acetylglucosaminidase (GlcNAc'ase) and the egg vitelline coat possesses complementary β-N-acetylglucosamine (β-GlcNAc) residues. In insects, the mechanisms of sperm-egg interactions are poorly understood and sperm-egg interactions have been studied in only two Dipteran species. *Drosophila* sperm possess fucosidase, mannosidase and β-N-hexosaminidase that have been proposed to recognize complementary fucose, mannose and β-N-acetylglucosamine residues on the surface surrounding the micropyle of the egg chorion (Intra et al., 2006; Intra et al., 2011; Perotti, 2001). The presence of multiple glycosidases suggests that multiple molecules may be involved in a complex sperm-egg recognition event (Perotti, 2001 Intra et al., 2006; Cattaneo et al., 2002). Similar to *Drosophila*, *Ceratitis capitata* sperm possess surface associated fucosidase, mannosidase and β-N-acetylglucosaminidase (Intra et al., 2011), although the presence of cognate sugars on the egg chorion was not determined.

The role of β-N-acetylhexosaminidase in sperm-egg recognition in *Drosophila melanogaster* is further supported by genetic evidence. In the *Casanova* mutant, the sperm head lacks β-N-acetylhexosaminidase and the flies are sterile due to the inability of the sperm to penetrate the egg (Perotti, 2001). The presence of β-N-acetylhexosaminidase on the sperm head is conserved across the *Drosophila* genus, and together with the finding that *Ceratitis capitata* sperm also have glycosidases in their plasma membrane and complementary glycoconjugates on the egg surface (Intra et al., 2011), suggests that these glycosidases may be necessary for successful fertilization in flies.

Despite these advances, the precise details of insect sperm-egg recognition are unknown in most insect taxa. In order to understand the molecular basis of fertilization in insects, it is critical to study multiple insect orders and to understand the key parameters that ultimately determine the molecular interactions that lead to successful sperm-egg recognition. In this study, we investigated the role of glycosidases in a Hemipteran, the North American semi-aquatic water strider *Aquarius remigis*. *A. remigis* provides an optimal system to examine the biochemical basis of sperm-egg recognition in Hemipterans since the sperm-egg interaction is likely to occur between receptors on the plasma membrane overlying the acrosomal region of the sperm and complementary ligands on an extracellular matrix surrounding the egg (Swanson and Vacquier, 2002; Vacquier and Moy, 1977; Vacquier et al., 1990; Burkin and Miller, 2000; Mengerink and Vacquier, 2001; Wassarman et al., 2001; Evans, 2000; Bleil and Wassarman, 1980a; Bleil and Wassarman, 1980b; Bleil and Wassarman, 1986; Vazquez et al., 1989; Yanagimachi, 1994) and *A. remigis* sperm contain an unusually long acrosomal process that is approximately 2.5 mm in length (Miyata et al., 2011). In most organisms, the acrosome makes up a minor fraction of the sperm and, therefore, acrosomal proteins, and the plasma membrane proteins surrounding the head, constitute a minor fraction of the total sperm protein. In contrast, the *A. remigis* sperm represents a markedly different situation that allows for copious quantities of protein to be extracted and examined for biochemical characterization.

In this study, two glycosidases, a mannosidase and an N-acetylglucosaminidase, from the *A. remigis* sperm plasma membrane were identified and characterized. As with

Dipterans, their presence on the sperm plasma membrane suggests that these molecules may play a role in sperm-egg interactions and the results from this study will help to elucidate the role that these molecules play in insect reproduction. This report represents the first study of molecules potentially involved in fertilization in Hemipterans.

Materials and methods

Materials

Sodium citrate, KI, Na_2HPO_4, NaH_2PO_4, NaCl, $CaCl_2$, $MnCl_2$, $MgCl_2$, and $ZnCl_2$ were purchased from Fisher Scientific (Waltham, MA) and $CoCl_2$ was purchased from Mallinckrodt (Paris, KY). Tris–HCl, Triton X-100, CHAPSO, CHAPS, 4-Nitrophenyl α-d-mannopyranoside (4NP-Man), 4-Nitrophenyl N-acetyl-β-d-glucosaminide (4NP-NAG), mannosidase and β-N-acetyl-glucosaminidase were purchased from Sigma–Aldrich (St. Louis, MO). Kifunensine and swainsonine were purchased from ToCris (Ellisville, MO). The Pierce BCA Protein assay kit was purchased from Thermoscientific (Waltham, MA).

Animals

A. remigis males and females were kept at room temperature in tanks partially filled with water. Frozen crickets were provided ad libitum daily and Styrofoam™ cups were placed in the water to provide shelter and oviposition sites. Tanks were cleaned daily and the water changed weekly. Males were isolated from females for two weeks or longer to ensure that sufficient amounts of mature sperm were present in the seminal vesicles.

Sperm collection

Adult males were euthanized using chloroform and the seminal vesicles were then dissected from the animals. Seminal vesicles were placed in phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM NaH_2PO_4, 135 mM NaCl, pH 7.2) and intact sperm were

removed from seminal vesicles according to previously published procedures (Miyata et al., 2011). Methods used to remove sperm for this study have been previously used for motility assays (Miyata et al., 2012).

Enzyme assays

The β-N-acetyl-glucosaminidase and mannosidase activity was quantified using the colorimetric substrates 4NP-NAG and 4NP-Man, respectively. Sperm extracts, or whole sperm, were incubated with an appropriate concentration of substrate in 100 mM NaH_2PO_4 held at an appropriate pH. The reaction mixture consisted of 100 mM NaH_2PO_4, various substrate concentrations (4NP-Man or 4NP-NAG) diluted in ddH_2O, and an appropriate enzyme concentration diluted in an extraction reagent (see below). All reactions were performed using either crude extract from sperm or commercially available enzymes (mannosidase and β-N-acetyl-glucosaminidase). The reaction mixtures were incubated for various times, depending on the specific assay, as indicated in the text. Absorbance changes from the hydrolyzed chromogenic 4-nitrophenyl substrate were measured using a spectrophotometer (DU-640, Beckmann Instruments, Brea, CA; $\lambda = 405$ nm). Background absorbance was determined by measuring these substrates alone over the same time course, and was subtracted from all assay data. The specifics of each assay are discussed below. All experiments were performed in triplicate.

Enzymatic activity with whole sperm

Whole intact sperm were incubated in 100 mM NaH_2PO_4 and either 10 mM 4NP-Man or 10 mM 4NP-NAG. The change in absorbance at 405 nm was measured for 20 h.

Background absorbance was determined by measuring substrate alone over the same time course, and was subtracted from all assay data.

Membrane extraction of glycosidases

Sperm were collected from the male seminal vesicles, as described above, and resuspended in 100 μL extraction agent (12 mM Triton X-100 in TBS; 100 mM CHAPSO in TBS; 0.6 M KI in TBS, or 1 M NaCl in TBS). The mixture was then vortexed for 2 min, incubated at 4 °C for 20–30 min with agitation, vortexed for an additional 30 s and then centrifuged at 2000 g in a Mini-Centrifuge (Thomas Scientific, Swedesboro, NJ) for 1 min. The supernatant was collected after pelleting. The procedure from the first vortexing step was repeated one time. To determine the efficiency of the extraction for a particular glycosidase, the sperm pellets and sperm extracts were incubated with either 0.625 mM or 1.25 mM of the appropriate chromogenic substrate in 100 mM NaH_2PO_4, pH 7.0, and then subjected to the appropriate glycosidase assays.

Protein concentration determination

Total protein concentrations of sperm extracts were determined using a bicinchoninic acid (BCA) assay. Solubilized protein was incubated in BCA solution for 2 h and the absorbance measured spectrophotometrically at 562 nm. BSA was used as a standard.

Determination of the optimal pH for enzyme activity

To determine the optimal pH for β-N-acetylglucosaminidase and mannosidase activity, the enzymes were incubated in 4-NP-NAG and 4-NP-Man in different buffers: 100 mM sodium citrate buffer for pH < 6, 100 mM NaH_2PO_4 buffer at pH 7 and 100 mM Tris–HCl buffer (TB) at ≥ pH 8. The concentrations of 4-NP-NAG and 4-NP-Man were 0.625 mM and 1.25 mM, respectively.

Determination of enzyme activity on the sperm surface

Michaelis–Menten coefficients (K_m and V_{max} values) were calculated from hyperbolic curves using a non-linear least square regression formula for each synthetic substrate. β-N-acetylglucosaminidase was incubated with its substrate at concentrations ranging from 0.15 mM to 5 mM and α-d-mannosidase was incubated with its substrate at concentrations ranging from 0.3 mM to 10 mM. Each experiment was repeated three times using sperm from three different animals.

Determination of thermal stability of glycosidases

To determine the thermal stability of the enzymes, whole sperm were boiled for 1 h, cooled to room temperature and then incubated in 4-NP-NAG or 4-NP-Man for 4 h. The absorbance of the released 4-NP was measured at 405 nm. Background absorbance was determined by measuring substrate alone over the same time course and was subtracted from all assay data.

Characterization of enzymes using individual divalent cations and class-specific enzyme inhibitors

To examine the effect of metal cations on sperm mannosidase and N-acetylglucosaminidase activity, sperm extracts were incubated in 0.625 mM 4-NP-NAG and 1.25 mM 4-NP-Man containing 0.5 mM of each cation used (Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+}) in 100 mM Hepes buffer for 20 h at RT. To determine if the mannosidase is a class I or class II enzyme, the effect of the inhibitors kifunesine (a Man I inhibitor) and swainsonine (a Man II inhibitor) were measured using two different concentrations of each inhibitor. Solubilized sperm were incubated in 0.625 mM 4-NP-NAG or 1.25 mM 4-NP-Man for 20 h.

Curve-fitting and statistical analysis

Sigma Plot 11.0 (Systat, Inc., San Jose, CA) was used for curve fitting for kinetic analysis and for plotting data. Differences between treatments were analyzed by Student's t-test or ANOVA and the Holm-Sidak pairwise multiple comparison test.

Results

Whole sperm from *A. remigis* possess both β-N-acetylglucosaminidase and α-D-mannosidase activity

In initial studies, whole sperm were incubated in either 4-NP-NAG or 4-NP-Man for 20 h and displayed an increase in absorbance at 405 nm (Table 1) and a color change was visible. The detection of β-N-acetylglucosaminidase and α-d-mannosidase activity in the absence of sperm permeabilization indicated that the enzymes are on the sperm surface and the catalytic site is oriented extracellularly making it available to bind glycoside ligands on the egg surface.

β-N-acetylglucosaminidase and α-d-mannosidase are sperm membrane glycosidases.

The majority of detectable enzymatic activity for both β-N-acetylglucosaminidase and α-d-mannosidase was abolished when sperm extracts were boiled for 1 h (data not shown). Under these conditions, β-N-acetylglucosaminidase retained approximately 8% of its activity and α-d-mannosidase retained approximately 2% of its activity (data not shown).

The association of β-N-acetylglucosaminidase and α-d-mannosidase with the sperm plasma membrane was studied using various extraction reagents (Fig. 1). Many peripheral proteins, but not integral proteins, are released from the plasma membrane by incubating cells in high ionic strength buffer without disrupting the lipid bilayer (Findlay, 1987, Shur and Neely, 1988, Downey and Lambert, 1994 and Fornes et al., 1996). Following incubation in high salt solutions (0.6 M KI or 1 M NaCl), greater than 70% of the β-N-acetylglucosaminidase activity was successfully released from the cell surface

(Fig. 1) indicating that sperm β-N-acetylglucosaminidase is likely a peripheral membrane protein. The α-d-mannosidase was also successfully released using KI (18%) and NaCl (63%) (Fig. 1) indicating that sperm α-d-mannosidase is likely also a peripheral membrane protein. A number of detergents effectively release integral membrane proteins (Shur and Neely, 1988, Hooper and Bashir, 1991 and Aviles et al., 1996) and the association of β-N-acetylglucosaminidase and α-d-mannosidase with the sperm plasma was further studied using such detergents. When incubated with either the detergent CHAPSO or Triton X-100, similar amounts of β-N-acetylglucosaminidase (76% and 82%, respectively) and α-d-mannosidase (74% and 31%, respectively) were released when compared to sperm that were incubated with high ionic strength buffers (Fig. 1). TBS released significantly less N-acetylglucosaminidase (57%) and mannosidase (0%) activity. Taken together, these results suggest that both *A. remigis* sperm β-N-acetylglucosaminidase and α-d-mannosidase are peripheral membrane proteins.

Effect of cations on mannosidase and N-acetylglucosaminidase activity

The effect of several metal cations on mannosidase and N-acetylglucosaminidase enzyme activity was studied at a concentration higher than the likely physiological concentration. This would ensure that enough of the cation was present to detect a change in activity. N-acetylglucosaminidase activity was not affected by Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+} or Zn^{2+} (Fig. 2). In contrast, mannosidase activity was inhibited by Mn^{2+} and Mg^{2+} and activated by Zn^{2+} but not by the other cations tested (Fig. 2).

A. remigis sperm possess a class II mannosidase

To determine whether *A. remigis* α-mannosidase is a class I or class II enzyme, the inhibitors kifunesine and swainsonine were used (Fig. 3A). Swainsonine was the most effective inhibitor, causing a significant reduction in enzyme activity at concentrations ≥60 nM (Fig. 3A). Swainsonine inhibits class II mannosidase activity. Swainosine inhibits both *Drosophila* Golgi α-mannosidase 2 and Golgi α-mannosidase 2b with K_is of 20 nM and 3 nM, respectively (Kuntz et al., 2008; Kuntz et al., 2010; Shah et al., 2003). Kifunesine was less effective and did not inhibit activity in the nanomolar to micromolar range (Fig. 3B). Kifunesine inhibits class 1 mannosidase activity and inhibits the human endoplasmic reticulum α-1,2-mannosidase I and Golgi Class I mannosidases IA, IB and IC with K_i s of 130 nM and 23 nM, respectively (Hering et al., 2005). Taken together, these results suggest that the *A. remigis* sperm mannosidase is a type II mannosidase.

β-N-acetylglucosaminidase and α-D-mannosidase have different optimal pHs and kinetic parameters

A. remigis sperm β-N-acetylglucosaminidase showed an optimal pH of 7 when 4-NP-NAG was used as a substrate (Fig. 4A). In contrast, β-N-acetylglucosaminidase from jack bean (*Canavalia ensiformis*) had a pH optimum of 6 when 4-NP-NAG was used as a substrate (Fig. 4A). *A. remigis* sperm α-d-mannosidase showed an optimal pH of 7 when 4-NP-Man was used as a substrate (Fig. 4B). This optimum was similar to an α-d-

mannosidase from *C. ensiformis* that had a pH optimum of 7 when 4-NP-Man was used as a substrate (Fig. 4B).

Reaction velocities were measured at increasing substrate concentrations using the substrates 4-NP-NAG and 4-NP-Man (Fig. 5 and Fig. 6). Both enzymes displayed a characteristic hyperbolic curve (Fig. 5 and Fig. 6A) that fit well to the Michaelis-Menten equation. The K_M and V_{max} values were determined using both non-linear curve fitting routines and from Lineweaver-Burk plots (Fig. 5 and Fig. 6B). For the sperm surface β-N-acetylglucosaminidase, the apparent K_m for 4-NP-NAG was 0.093 ± 0.01 mM, the V_{max} was 153.8 ± 2.97 pmol/min, and the Hill coefficient was 0.96 ± 0.63 (Fig. 5C). For the α-d-mannosidase, the apparent K_m for 4-NP-Man was 2.36 ± 0.19 mM, the V_{max} was 27.49 ± 0.88 pmol/min, and the Hill coefficient was 0.94 ± 0.18 (Fig. 6C).

Discussion

Most animal systems studied to date provide evidence for receptor mediated recognition and binding between sperm surface receptor and complementary ligand in the extracellular matrix that surrounds the egg (Downey and Lambert, 1994; Habermann and Sinowatz, 2011; Sinowatz et al., 1998; Focarelli et al., 2001). Some of the sperm receptors possess intrinsic enzymatic activity by which they can be characterized, although, in fertilization, they are thought to function by recognizing substrates without completing their enzymatic cycle (Tulsiani et al., 1997; Godknecht and Honegger, 1991; Matsumoto et al., 2002; Downey and Lambert, 1994; Perotti, 2001; Pasini et al., 1999; Intra et al., 2006; Intra et al., 2011; Cattaneo et al., 1997). In many taxa, such receptors are thought to act as sperm surface lectin-like molecules that recognize glycoconjugates on the egg coat leading to sperm-egg binding (Tulsiani et al., 1997; Martinez et al., 2000; Miranda et al., 2000; Mengerink and Vacquier, 2001; Rodeheffer and Shur, 2002; Koyanagi and Honegger, 2003).

With the exception of some Dipteran species, little is known regarding receptors in insect sperm that are involved in sperm-egg interactions. Most cells possess glycosidases as soluble lysosomal proteins that are involved in hydrolysis of complex carbohydrates. However, in this study, we detected both a β-N-acetylglucosaminidase (EC 3.2.1.52) and an α-mannosidase (EC 3.2.1.24) on the surface of sperm from the hemipteran *A. remigis*. Significantly, both of these enzymes had catalytic sites oriented extracellularly, indicating their ability to recognize complementary substrates outside of the cell. Similar enzymes have also been reported in the sperm of mollusks (Focarelli

et al., 2001), ascidians (Hoshi et al., 1983; Hoshi et al., 1985; Hoshi, 1984; Matsumoto et al., 2002; Koyanagi and Honegger, 2003), amphibians (Martinez et al., 2000), rodents (Tulsiani et al., 1989; Cornwall et al., 1991; Abascal et al., 1998), and humans (Tulsiani et al., 1990; Khunsook et al., 2003; Zitta et al., 2006), as well as *Drosophila* (Perotti, 2001; Intra et al., 2006; Cattaneo et al., 2002), and *Ceratitis* (Intra et al., 2011).

Drosophila sperm possess a mannosidase and two β-N-acetylglucosaminidase isoforms restricted to areas of the plasma membrane overlying the acrosome and the posterior tail (Cattaneo et al., 2002; Perotti, 2001). The mannosidase was 317 kDa and both of the β-N-acetylglucosaminidase isoforms were 158 kDa (Cattaneo et al., 2002). An ion exchange column was used to determine that there were two β-N-acetylglucosaminidase isoforms because the two isoforms were eluted at different sodium chloride concentrations (Cattaneo et al., 2002). These two isoforms are named Hex1 and Hex2. Hex1 is likely a heterodimer; whereas, Hex2 is likely a homodimer (Cattaneo et al., 2002). Although, it is not uncommon for β-N-acetylglucosaminidase isoforms and dimers to exist, there is currently no evidence that either multiple isoforms or dimers are present in *A. remigis* sperm or are affecting glycosidase activity. Future kinetic studies involving natural or synthetic substrates may be helpful for identifying the presence of different isoforms on the *A. remigis* sperm surface. Further evidence that glycosidases may be involved sperm-egg recognition is provided by the *Drosophila* casanova mutant that lacks β-N-acetylglucosaminidase overlying the sperm acrosome and is infertile because sperm are unable to penetrate the egg (Perotti et al., 2001). Moreover, *Drosophila* eggs possess the glycoconjugates recognized by mannosidase and β-N-

acetylglucosaminidase (Intra et al., 2009). As with other animals that have been investigated to date, the initial site of sperm-egg interactions in insects is predicted to be on the plasma membrane overlying the sperm head, and specifically over the acrosomal region. Given the small number of sperm that are available from most insects, combined with the small surface area overlying the head region, detailed biochemical studies in these systems would be difficult if not prohibitive.

Because of their large size and, specifically, because half of the length of the sperm is composed of the acrosomal process (Tandler and Moriber, 1966; Miyata et al., 2011; Miyata et al., 2012), *A. remigis* provides a good system to characterize proteins on the sperm surface, including those that may be involved in sperm-egg interactions. Given their localization, combined with what it is known about other sperm surface enzymes in other systems, it is likely that the glycosidases studied here have a physiological function in *A. remigis* reproduction. To our knowledge, this study comprises the first biochemical characterization of sperm components of any Heteropteran, and it will be important to determine the localization of conjugate glycans on the egg chorion in future studies. Previous studies on flies have indicated that glycosidases from sperm of *Ceratitis capitata* and *D. melanogaster* (Intra et al., 2011; Cattaneo et al., 2002; Intra et al., 2006) are transmembrane proteins. In contrast, we found that both the *A. remigis* sperm surface mannosidase and N-acetylglucosaminidase are released from the sperm plasma membrane by high salt solutions, indicating that these glycosidases are likely peripheral membrane proteins. We further found that the *A. remigis* sperm plasma membrane possesses a type II mannosidase, as indicated by its inhibition by swainsonine, but not

kifunesine. This result indicates that the mannosidase would cleave 1,3-mannose linkages and not 1,2-mannose or 1,6-mannose linkages (Tulsiani et al., 1982). In contrast to the *A remigis* sperm mannosidase, *Ceratitis*, human, and rat sperm mannosidase activity is not inhibited by swainsonine. *A. remigis* mannosidase is also activated by Zn^{2+} when compared with Ca^{2+}, Mn^{2+} or Mg^{2+}, but is not affected by Co^{2+}. In contrast, *Ceratitis* sperm mannosidase is activated by Zn^{2+}, Mn^{2+}, and Co^{2+}, inhibited by Ca^{2+}, and unaffected by Mg^{2+} at concentrations higher than those used in the present study (Intra et al., 2011).

The pH optima of the *A. remigis* sperm glycosidases may also provide important clues about its physiological role in reproduction. The *A. remigis* sperm plasma membrane α-d-mannosidase has a pH optimum of approximately 7, compared to the *D. melanogaster* and *Ceratitis* sperm plasma membrane α-d-mannosidases that have optima of pH 4 (Cattaneo et al., 1997) and pH 4.5 (Intra et al., 2011), respectively. Similarly, the *A. remigis* sperm plasma membrane β-N-acetylglucosaminidase has a pH optimum of 7 (compared to the lysosomal form with a pH optimum of 6), which is more basic than the pH optima of the sperm β-N-acetylglucosaminidase from *D. melanogaster* (pH 6–6.5; Cattaneo et al., 1997) or *Ceratitis* (pH 5; Intra et al., 2011). The pH optimum of β-N-acetylglucosaminidase in *A. remigis* sperm is most similar to Hex2 of *D. melanogaster* (Cattaneo et al., 2002). Furthermore, the presence of only one pH optimum as opposed to a bimodal pH optimum curve argues strongly against the presence of multiple isoforms being present on *A. remigis* sperm. This is further supported by analysis of both isoforms of β-N-acetylglucosaminidase in a number of

Drosophila species where it was found that the pH optima for Hex1 and Hex2 were very similar in all *Drosophila* species studied (Intra et al., 2009). The rat sperm surface mannosidase that is thought to play a role in sperm-egg interactions has an optimal pH of ~6.5, but maintains most of its activity at pH 7 (Tulsiani et al., 1995). These optimal pH values may provide important clues regarding the local reproductive environment that may maximize their function in reproductive processes.

Characterizing the enzymatic activity to determine maximal turnover rates and affinities may also provide clues regarding the role of these enzymes as potential binding molecules. The K_M from sperm membrane α-d-mannosidase was 580 ± 47 μM in *Ceratitis* (Intra et al., 2011), 2520 ± 240 μM in *Drosophila* (Cattaneo et al., 2002), and 2617 ± 3750 μM in *A. remigis* (this study). In contrast, the K_M for the sperm plasma membrane β-N-acetylglucosaminidase displayed higher affinities than the α-d-mannosidase in all three of these insect species with values of 106 ± 15 μM for *Ceratitis* (Intra et al., 2011), 260 ± 31 μM for Drosophila (Cattaneo et al., 2002), and 90 ± 25 μM for *A. remigis.* In *Drosophila*, mannosidase sharply departs from normal Michaelis–Menten kinetics indicating the presence of allosteric interactions but there is no evidence of allostery in the *Drosophila* β-N-acetylglucosaminidase (Cattaneo et al., 2002). None of these *A. remigis* enzymes displayed cooperative binding indicating that there is no change in affinity with increasing substrate concentration and that even if dimers are present, they are not affecting the enzyme's ability to bind and process a substrate.

It has been hypothesized that glycosidases are involved in sperm-egg interaction. If so, presence of multiple glycosidases on the sperm surface would suggest that there

may be multiple receptors involved in sperm-egg recognition and that combinations of these receptors, along with variations in glycoconjugate densities on the egg extracellular surface may influence sperm-egg interactions during fertilization. Should glycosidases be involved in a recognition event, multiple classes of binding moieties would allow for the possibility of a level of cooperativity that extends beyond the nature of individual receptors and ligands and may reflect on both the class and distribution of complementary ligands on the chorion surrounding the *A. remigis* egg. Future studies will focus on the role of these enzymes in reproduction including the distribution of these receptors on the sperm surface as well as the distribution of complementary glycoconjugates on the egg coats in *A. remigis*.

References

Abascal I., Skalaban S., Grimm K., Aviles M., Martianez-Menarguez J., Castells M., Ballesta J. and Aldaheff J. (1998). Alteration of the isoform composition of plasma-membrane-associated rat sperm alpha-L-fucosidase during late epididymal maturation: comparative characterization of the acidic and neutral isoforms. *J. Biochem*. 333: 201-207.

Aviles M., Abascal I., Martinez-Menarguez J., Castells M., Skalaban S., Ballesta J. and Aldaheff J. (1996). Immunocytochemical localization and biochemical characterization of a novel plasma membrane-associated, neutral pH optimum alpha-L-fucosidase from rat testis and epididymal spermatozoa. *J. Biochem*. *318*: 821-831.

Bleil J. and Wassarman P. (1980). Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell,* 20: 873-882.

Bleil J. and Wassarman P. (1986). Autoradiographic Visualization of the Mouse Egg's Sperm Receptor Bound to Sperm. *J. Cell Biol*. 102: 1363-1371.

Bleil J. and Wassarman, P. (1980). Synthesis of zona pellucida proteins by denuded and follicle-enclosed mouse oocytes during culture in vitro. *PNAS*. 77: 1029-1033.

Burkin H. and Miller D. (2000). Zona pellucida protein binding ability of porcine sperm during epididymal maturation and the acrosome reaction. *Dev. Biol*. 222: 99-109.

Cattaneo F., Ogiso M., Hoshi M., Perotti M. and Pasini M. (2002). Purification and characterization of the plasma membrane glycosidases of *Drosophila melanogaster* spermatozoa. *Insect Biochem. Mol. Biol.* 32: 929-941.

Cattaneo F., Pasini M. and Perotti M. (1997). Glycosidases are present on the surface of *Drosophila melanogaster* spermatozoa. *Mol. Reprod. Dev.* 48 (2): 276-281.

Chiu P.C., Wong B.S., Chung M.K., Lam K.K., Pang R.T., Lee K.F., Sumitro S.B., Gupta S.K. and Yeung, W.S. (2008). Effects of native human zona pellucida glycoproteins 3 and 4 on acrosome reaction and zona pellucida binding of human spermatozoa. *Biol. Reprod.* 79: 869-877.

Claw K.G. and Swanson W.J. (2012). Evolution of the egg: new findings and challenges. *Annu. Rev. Genome. Hum. Genet.*13: 109–125.

Cornwall G., Tulsiani D. and Orgebin-Crist M. (1991). Inhibition of the mouse sperm surface alpha-D-mannosidase inhibits sperm-egg binding in vitro. *Biol. Reprod*. 44 (5): 913-921.

Demott R.P., Lefebvre R. and Suarez S.S. (1995). Carbohydrates mediate the adherence of hamster sperm to oviductal epithelium. *Biol. Reprod*. 52: 1395-403.

Dobrinski I., Ignotz G.G., Thomas P.G. and Ball B.A. (1996). Role of carbohydrates in the attachment of equine spermatozoa to uterine tubal (oviductal) epithelial cells in vitro. *Am. J. Vet. Res.* 57: 1635-9.

Downey J. and Lambert C. (1994). Attachment of the ascidian sperm surface egg receptor N- acetylglucosaminidase to the cell membrane. *Mol. Reprod. Dev*. 38 (4): 453-458.

Evans J. (2000). Getting sperm and egg together: things conserved and things diverged. *Biol. Reprod.* 63: 355-360.

Findlay J. 1987. The isolation and labelling of membrane proteins and peptides. In Findlay J. and Evans W. Biological Membranes: A Practical Approach (p. 179). Washington DC: IRL Press.

Focarelli R., La Sala G., Balasini M. and Rosati, F. (2001). Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatus* model. *Cells Tissues Organs*. 168: 76-81.

Fornes M., Barbieri M. and Bertini F. (1996). Electrophoretic Analysis of Protein Selectively Extracted with Chaotropic Agents from Membranes of Rat Sperm. *Biocell.* 20: 111-121.

Godknecht A. and Honegger T. (1991). Isolation, Characterization, and Localization of a Sperm-Bound N-Acetylglucosaminidase that Is Indispensable for Fertilization in the Ascidian, *Phallusia mammillata*. *Dev. Biol.* 143: 398-407.

Green C.E., Bredl J., Holt W.V., Watson P.F. and Fazeli A. (2001). Carbohydrate mediation of boar sperm binding to oviductal epithelial cells in vitro. *Reproduction* 122: 305-15.

Habermann F. and Sinowatz F., 2011. Glycobiology of Fertilization and Early Embryonic Development. In Gabius, H., *The Sugar Code: Fundamentals of Glycosciences.* Weinheim: Wiley-VCH Verlag GmbH and Co KGaA, Weinheim.

Harper M.J. (1973). Relationship between sperm transport and penetration of eggs in the rabbit oviduct. *Biol. Reprod*. 8: 441-50.

Hering K., Karaveg K., Moreman K. and Pearson W. (2005). A Practical Synthesis of Kifunesine Analogues as Inhibitors of Endoplasmic Reticulum α-Mannosidase I. *J. Organic Chem.* 70 (24): 9892-9904.

Hooper and Bashir. (1991). Glycosyl-phosphatidylinositol-anchored membrane proteins can be distinguished from transmembrane polypeptide-anchored proteins by differential solubilization and temperature-induced phase separation in Triton X-114. *J. Biochem.* 280: 745-751.

Hoshi M. (1984). *Roles of sperm glycosidases and proteases in the ascidian fertilization. In Advances in Invertebrate Reproduction.* (W. Engels, Ed.) Amsterdam: Elselvier.

Hoshi M. (1986). Sperm glycosidases as a plausible mediator of sperm binding to the vitelline envelope in ascidians. In J. Hedrick, *The Molecular and Cellular Biology of Fertilization* (pp. 251-260). New York: Plenum Press.

Hoshi M., De Santis R., Pinto M., Cotelli F. and Rosati F. (1983). Is sperm a-L-fucosidase responsible for sperm-egg binding in *Ciona intestinalis*. *The Sperm Cell.* 107-110.

Hoshi M., De Santis R., Pinto M., Cotelli F. and Rosati F. (1985). Sperm glycosidases as mediators of sperm–egg binding in the ascidians. *Zool. Sci.* 2:65-69.

Hunter R.H. (1981). Sperm transport and reservoirs in the pig oviduct in relation to the time of ovulation. *J. Reprod. Fertil*. 63: 109-17.

Hunter R.H. and Nichol R. (1983). Transport of spermatozoa in the sheep oviduct: preovulatory sequestering of cells in the caudal isthmus. *J. Exp. Zool.* 228: 121-8.

Intra J., Caro D., Perotti M., Pasini M. (2011). Glycosidases in the plasma membrane of *Ceratitis capitata* spermatozoa. *Insect Biochem. and Mol. Biol.* 41: 90-100.

Intra J., Cenni F. and Perotti M. (2006). An a-L-Fucosidase Potentially Involved in Fertilization Is Present on Drosophila Spermatozoa Surface. *Mol. Reprod. Dev*. 73: 1149-1158.

Intra J., Cenni F., Pavesi G., Pasini M. and Perotti M. (2009). Interspecific Analysis of the Glycosidases of the Sperm Plasma Membrane in Drosophila. *Mol. Reprod. Dev*. 76: 85-100.

Khunsook S., Bean B., McGowan S. and Alhadeff J. (2003). Purification and characterization of plasma membrane-associated human sperm alpha-L-fucosidase. *Biol. Reprod.* 68: 709-716.

Koyanagi R. and Honegger T. (2003). Molecular cloning and sequence analysis of an ascidian egg β-N-acetylhexosaminidase with a potential role in fertilization. *Dev. Growth Differ*. 45:209-218.

Kuntz D., Nakayama S., Shea K., Hori H., Uto Y., Nagasawa H. and Rose D.R. (2010). Structural investigation of the binding of 5-substituted swainsonine analogues to Golgi alpha-mannosidase II. *Chem. Biochem*. 11: 673-80.

Kuntz D., Tarling C., Withers S. and Rose D. (2008). Structural analysis of Golgi alpha-mannosidase II inhibitors identified from a focused glycosidase inhibitor screen. *Biochemistry*. 47 (38): 10058-10068.

Lambert C. (1989). Ascidian sperm penetration and the translocation of a cell surface glycosidase. *J. Exp. Zool.* 249: 308-315.

Martinez M., Martelotto and Cabada M. (2000). Purification and biological characterization of N-acetyl β-d-glucosaminidase from Bufo arenarum spermatozoa. *Mol. Reprod. Dev*. 57: 194-203.

Matsumoto M., Hirata J., Hirohashi N. and Hoshi M. (2002). Sperm-Egg Binding Mediated by Sperm Alpha-L-Fucosidase in The Ascidian *Halocynthia roretzi*. *Zool. Sci*. 19: 43-48.

Mengerink K. and Vacquier V. (2001). Glycobiology of Sperm-Egg Interactions in Deuterostomes. *Glycobiology.* 11 (4): 37-43.

Miranda P., Gonzalez Echeverria F., Blaquier J., Mahuran D. and Tezon J. (2000). Evidence for the participation of b-hexosaminidase in human sperm-zona pellucida interaction in vitro. *Mol. Hum. Reprod.* 6: 699-706.

Miyata H., Noda N., Fairbairn D., Oldenbourg R. and Cardullo R. (2011). Asembly of the Fluorescent Acrosomal Matrix and its Fate in Fertilization in the Water Strider, *Aquarius remigis. J. Cell Physiol*. 226 (4): 999-1006.

Miyata H., Thaler C., Haimo L. and Cardullo R. (2012). Protease Activation and the Signal Transduction Pathway Regulating Motility in Sperm from the Water Strider *Aquarius remigis*. *Cytoskeleton*. 69: 207-220.

Overstreet J.W. and Cooper G.W. (1978). Sperm transport in the reproductive tract of the female rabbit: II. The sustained phase of transport. *Biol. Reprod*. 19: 115-32.

Pang P.C., Chiu P.C., Lee C.L., Chang L.Y., Panico M., Morris H.R., Haslam S.M., Khoo K.H., Clark G.F., Yeung W.S. and Dell A. (2011). Human sperm binding is mediated by the sialyl-Lewisx oligosaccharide on the zona pellucida. *Science*. 333: 1761–1764.

Pasini M., Cattaneo F., Pinto M., De Santis R. and Perotti M. (1999). Plasma membrane

association and preliminary characterization of Drosophila sperm surface glycosidases. *Mol. Reprod. Dev.* 52: 166-173.

Perotti M. C. (2001). Male sterile mutant casanova gives clues to mechanisms of sperm-egg interactions in *Drosophila melanogaster*. *Mol. Reprod. Dev*. 60 (2): 248-259.

Rodeheffer C. and Shur B. (2002). Targeted mutations in β1,4 galactosyltransferase I reveal its multiple cellular functions. *Biochim. Biophys. Acta. Reviews. 1573*: 258-270.

Seager S., Slabaugh M. (2010). *Organic and Biochemistry for Today* (7th edition ed.).

Shah N., Kuntz D. and Rose D. (2003). Comparison of kifunensine and 1-deoxymannojirimycin binding to class I and II alpha-mannosidases demonstrates different saccharide distortions in inverting and retaining catalytic mechanisms. *Biochemistry*. 42 (47): 13812-6.

Shur B. and Neely C. (1988). Plasma membrane association, purification and characterization of mouse sperm ß1,4 galactosyltransferase. *J. Biol. Chem*. 263: 17706-17714.

Sinowatz F., Plendl J. and Kolle S. (1998). Protein-carbohydrate Interactions During Fertilization. *Acta anat.* 161: 196-205.

Swanson W. and Vacquier V. (2002). Reproductive Protein Evolution. *Ann. Rev. Ecol. Syst.* 33: 161-179.

Suarez S.S. (1987). Sperm transport and motility in the mouse oviduct: observations in situ. *Biol. Reprod*. 36: 203-10.

Tandler B. and Moriber L. (1966). Microtubular structures associated with the acrosome during spermiogenesis in the Water-strider, *Gerris remigis* (Say). *J. Ultrastruct. Res.* 14: 391-404.

Tulsiani D., Harris T. and Touster O. (1982). Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of Golgi mannosidase II. *J. Biol. Chem*. 257 (14): 7936-7939.

Tulsiani D., NagDas S., Skudlarek M. and Orgebin-Crist M. (1995). Rat sperm plasma membrane mannosidase: localization and evidence for proteolytic processing during epididymal maturation. *Dev. Biol*. 167 (2): 584-595.

Tulsiani D., Skudlarek M. and Orgebin-Crist M. (1990). Human sperm plasma membranes possess alpha-D-mannosidase activity but no galactosyltransferase activity. *Biol. Reprod*. 42 (5-6): 843-858.

Tulsiani D., Skudlarek M. and Orgebin-Crist M. (1989). Novel C -D-Mannosidase of Rat Sperm Plasma Membranes: Characterization and Potential Role in Sperm-Egg Interactions. *J. Cell Biol*. 109: 1257-1267.

Tulsiani D., Yoshida-Komiya H. and Araki Y. (1997). Mammalian Fertilization: A Carbohydrate-Mediated Event. *Biol. Reprod.* 57: 487-494.

Vacquier V. and Moy G. (1977). Isolation of bindin: The protein responsible for adhesion of sperm to sea urchin eggs. *PNAS.* 74: 2456-2460.

Vacquier V., Carner K. and Stout C. (1990). Species-specific sequences of abalone lysin, the sperm protein that creates a hole in the egg envelope. *PNAS.* 87: 5792-5796.

Vazquez M., Phillips D. and Wassarman P. (1989). Interaction of mouse sperm with purified sperm receptors covalently linked to silica beads. *J. Cell Sci*. 92: 713-722.

Wagner A., Ekhlasu-Hundreiser M., Hettel C., Petrunkina A., Waberski D., Nimtz M. and Topfer-Petersen E. (2002). Carbohydrate-based interactions of oviductal sperm reservoir formation-studies in the pig. *Mol. Reprod. Dev*. 61: 249-57.

Wassarman P., Jovine L. and Litscher E. (2001). Profile of Fertilization in Mammals. *Nature Cell Biol.* 3: E59-E64.

Wilmut I. and Hunter R.H. (1984). Sperm transport into the oviducts of heifers mated early in oestrus. Reprod. Nutr. Dev. 24: 461-8.

Yanagimachi R. (1994). *Mammalian fertilization. In The Physiology of Reproduction.* New York: Raven.

Yanagimachi R. and Chang M.C. (1963). Sperm Ascent Through The Oviduct Of The Hamster And Rabbit In Relation To The Time Of Ovulation. *J. Reprod. Fertil.* 6: 413-20.

Zitta K., Werthheimer E. and Miranda P. (2006). Sperm N-acetylglucosaminidase is involved in primary binding to the zona pellucida. *Mol. Hum. Reprod*. 12 (9): 557-563.

Figures and Tables

Figure 4.1. Differential release of β-N-acetylglucosaminidase (black) and α-mannosidase (gray) activities from the *A. remigis* sperm surface. Glycosidase activity was extracted from the sperm surface as described in the text and activity is expressed as the percent activity released into the soluble fraction. Differences between treatments were analyzed by Student's t-test ($P<0.05$). β-N-acetylglucosaminidase (black) and α-mannosidase (gray) activities were compared to each other. Error bars represent standard error of the mean. N=2.

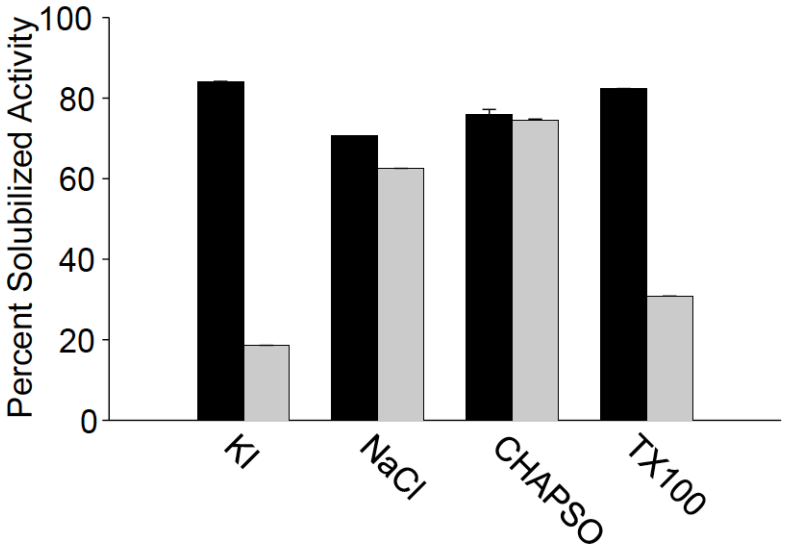


Figure 4.2A. Effect of the inhibitors (A) Swainsonine on mannosidase activity. Enzyme activity was measured by the standard β-N-acetylglucosaminidase and α-mannosidase assays using p-NP-(GlcNAc)2 and p-NP-Man as substrates for 20 h at RT. N-acetylglucosaminidase (NAG) was used as a negative control. The positive control was mannosidase from the *Canavalia ensiformis* (Con). Swainsonine (sw) and kifunesine (kif). Asterisks denote statistically different groups ($P<0.05$) as determined by Student's t-test. Error bars represent standard error of the mean. N=2.

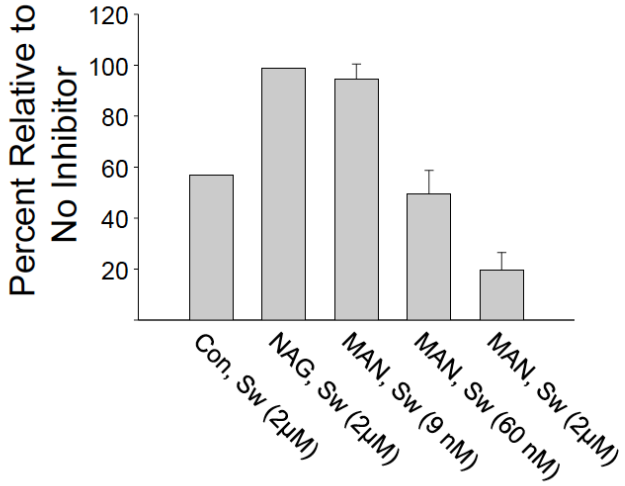


Figure 4.2B. Effect of the inhibitors (B) Kifunesine on mannosidase activity. Enzyme activity was measured by the standard β-N-acetylglucosaminidase and α-mannosidase assays using p-NP-(GlcNAc)2 and p-NP-Man as substrates for 20 h at RT. N-acetylglucosaminidase (NAG) was used as a negative control. The positive control was mannosidase from the *Canavalia ensiformis* (Con). Swainsonine (sw) and kifunesine (kif). Asterisks denote statistically different groups ($P<0.05$) as determined by Student's t-test. Error bars represent standard error of the mean. N=2.
Figure 2b

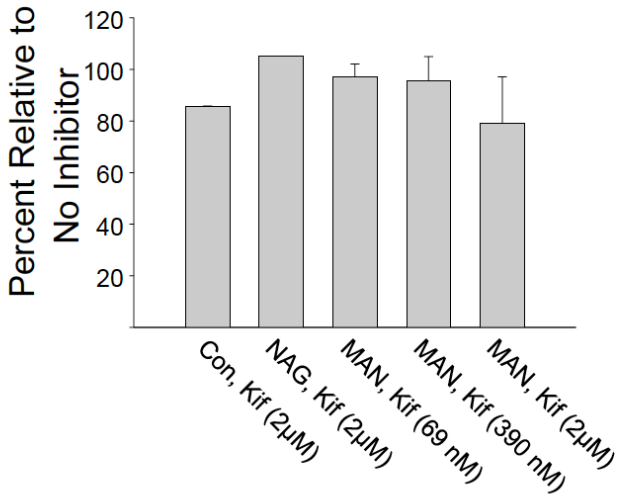


Figure 4.3. Effect of cations on β-N-acetylglucosaminidase (black) and α-mannosidase (gray) activity. Enzyme activity was measured by standard N-acetylglucosaminidase and mannosidase assays using p-NP-(GlcNAc)2 and p-NP-Man as substrates for 20 h at RT. β-N-acetylglucosaminidase activity was not significantly affected by the presence of cations when analyzed using ANOVA. When analyzed by ANOVA and the Holm-Sidak pairwise multiple comparison test, Mn^{2+} inhibits mannosidase activity when compared to Zn^{2+} (p= 0.013). When analyzed by ANOVA and the pairwise multiple comparison procedure, Holm-Sidak, Mg^{2+} inhibits mannosidase activity when compared to Zn^{2+} (p=0.025). Asterisks denote statistically different groups. Differences between treatments were analyzed by ANOVA and the Holm-Sidak pairwise multiple comparison test. β-N-acetylglucosaminidase (black) and α-mannosidase (gray) activities were only compared to each other. Error bars represent standard error of the mean. N=2.

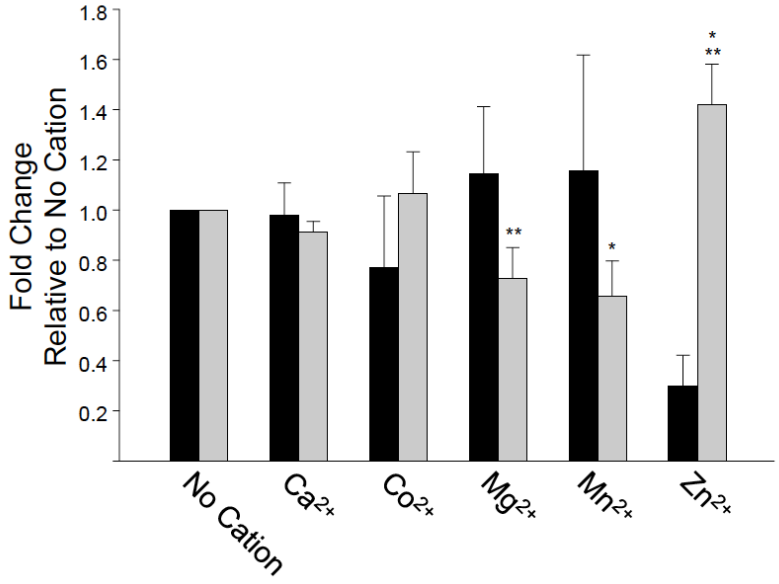


Figure 4.4A. Effects of pH on N-acetylglucosaminidase and α-mannosidase activity. (A) N-acetylglucosaminidase activity was measured by standard assay using p-NP-(GlcNAc)2 as the substrate for 20 h at RT. Filled circles represent commercially purchased enzyme extracted from *Canavalia ensiformis*. Unfilled circles represent enzyme activity in the sperm plasma membrane extract. N=2.

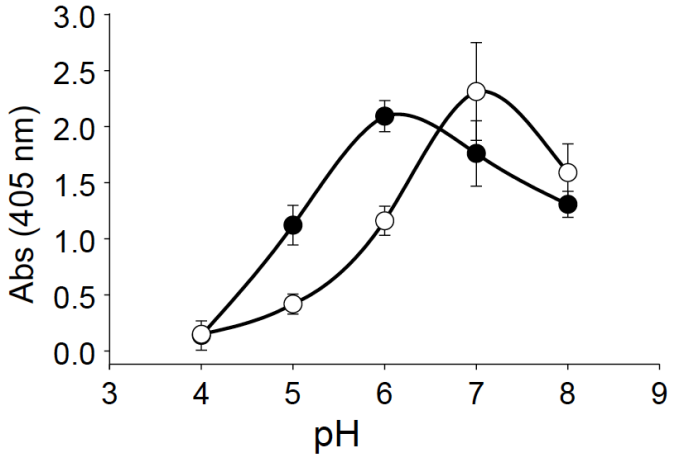


Figure 4.4B. Effects of pH on N-acetylglucosaminidase and α-mannosidase activity. (B) The α-mannosidase activity was measured by the standard assay using p-NP-Man as the substrate for 20 h at RT. Buffers: pH 4-6 sodium citrate; pH 7 sodium phosphate; pH 8 Tris-HCl. Error bars represent standard error of the mean. N=2.

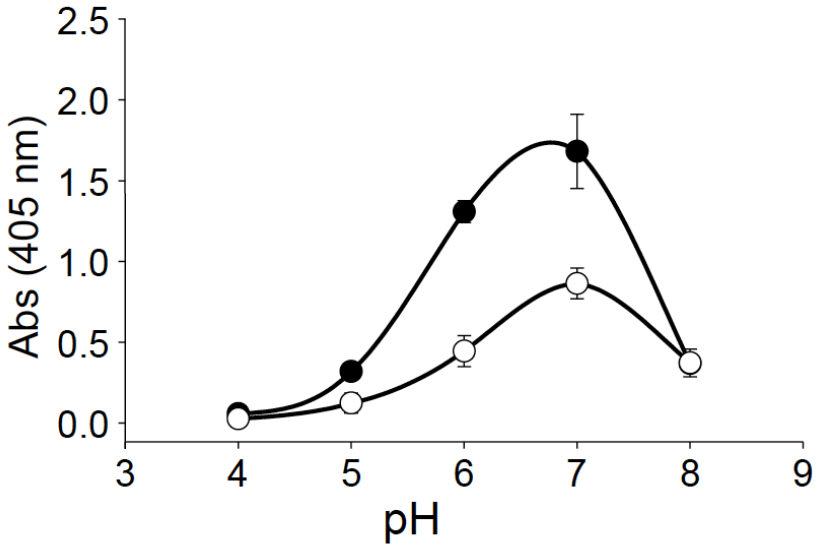


Figure 4.5A. Kinetic analysis of β-N-acetylglucosaminidase. Michaelis–Menten (A) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl N-acetyl- β-D-glucosaminide as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

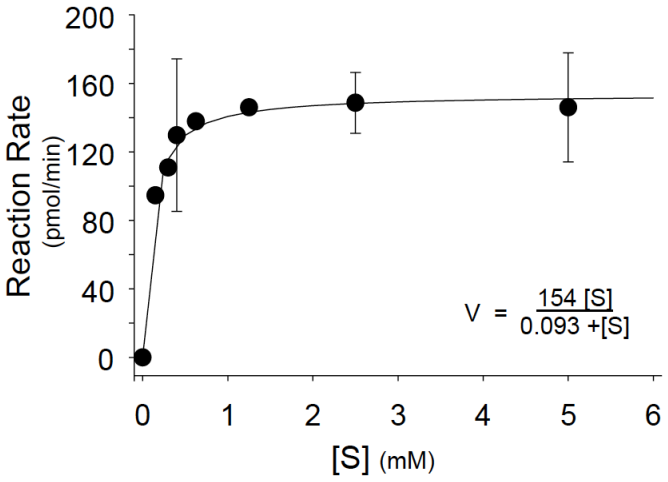


Figure 4.5B. Kinetic analysis of β-N-acetylglucosaminidase. Lineweaver Burke (B) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl N-acetyl- β-D-glucosaminide as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

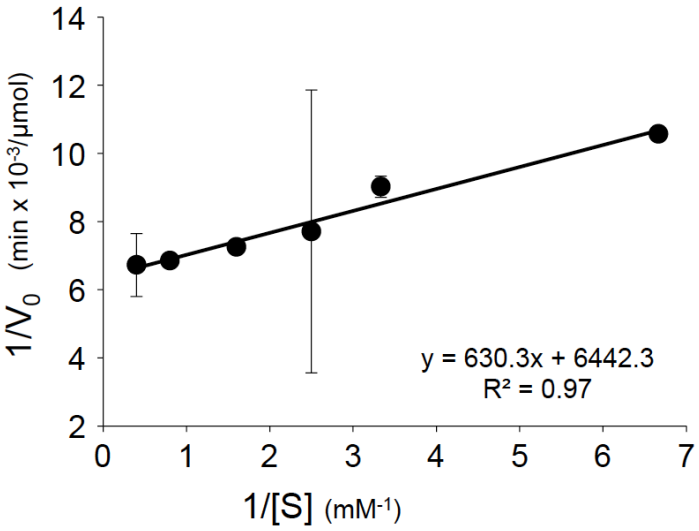


Figure 4.5C. Kinetic analysis of β-N-acetylglucosaminidase. Hill (C) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl N-acetyl- β-D-glucosaminide as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

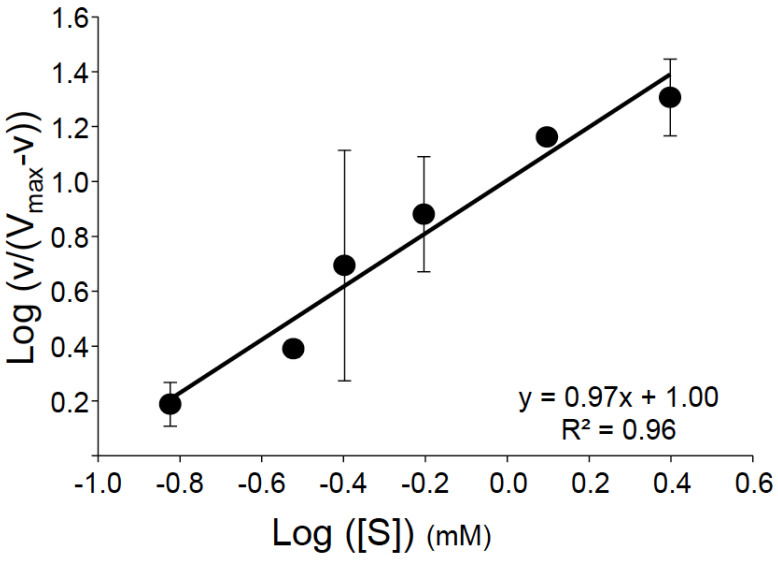


Figure 4.6A. Kinetic analysis of α-D-mannosidase. Michaelis–Menten (A) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl α -D-mannopyranoside as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

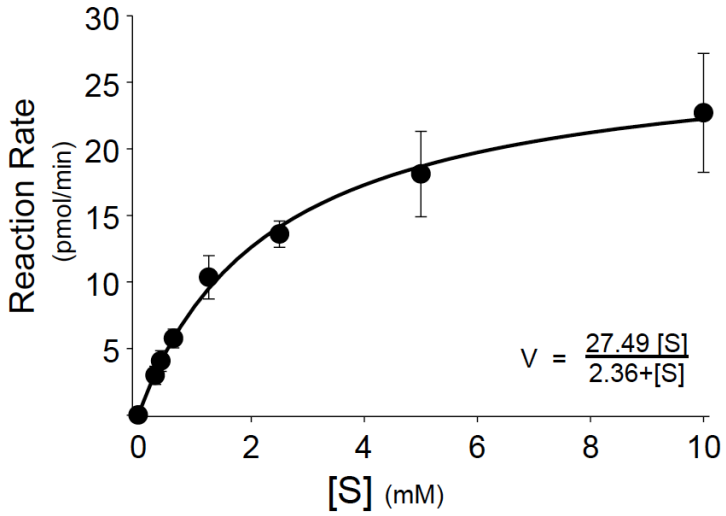


Figure 4.6B. Kinetic analysis of α-D-mannosidase. Lineweaver Burke (B) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl α -D-mannopyranoside as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

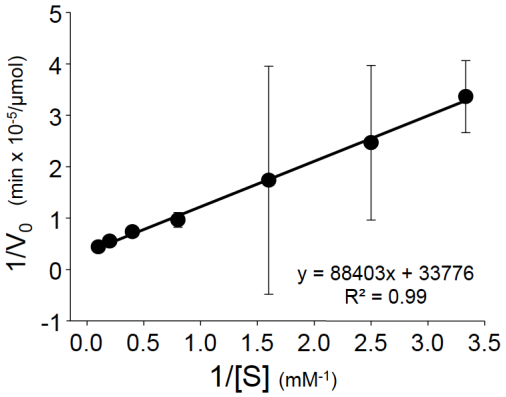


Figure 4.6C. Kinetic analysis of α-D-mannosidase. Hill (C) plots were generated from assays using the indicated concentrations of sperm membrane extract and 4-Nitrophenyl α -D-mannopyranoside as the substrate at pH 7. Error bars represent standard error of the mean. N=2.

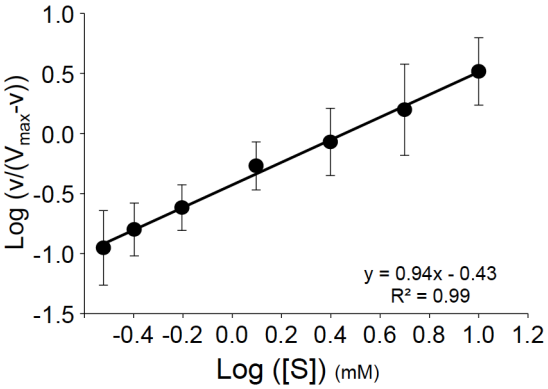


Table 4.1. Activity of β-N-acetylglucosaminidase and α-mannosidase in whole sperm relative to commercially available enzymes.

NAG'ase	Absorbance (405 nm)	MAN'ase	Absorbance (405 nm)
No enzyme	-0.1016	No enzyme	0.0004
+ enzyme[a]	0.1277	+ enzyme[c]	0.0634
whole sperm[b]	0.4453	whole sperm[b]	0.0481

[a] 0.1 units NAG'ase for 20 h at RT

[b] sperm from 2 seminal vesicles, total protein approx. 10 μg

[c] 0.1 units MAN'ase for 20 h at RT

CHAPTER 5

Testicular Transcription of Neuropeptide Receptors Potentially Involved in Regulating Circadian Rhythms in *Culex* Mosquitoes

Testicular Transcription of Neuropeptide Receptors Potentially Involved in Regulating Circadian Rhythms in *Culex* mosquitoes

Abstract

There is evidence in several moth species that movement of sperm from the testes to seminal vesicles is controlled by circadian rhythms (Giebultowicz et al., 1988; Giebultowicz et al., 1992). In *Pectinophora gossypiela* sperm movement is periodic (Thorson and Riemann, 1977), while in *Anagasta keuhnilla,* sperm are released in a rhythmic pattern that appears to be entrained by a light-dark cycle (Riemann et al., 1974). In both *Lymantria dispar* and *Spodoptera littoralis,* circadian rhythms are involved in movement of sperm from testes to seminal vesicles (Giebultowitcz et al., 1988; Bebas et al., 2001). In *Lymantria dispar,* sperm is moved rhythmically from the testes and this movement is modulated by temperature and photoperiod (Giebultowitcz et al., 1988). In mammals, Neuropeptide Y receptor (NPYR), Somatostatin receptor (SSR) and serotonin receptor (5HTR) are involved in circadian rhythms (Pickard and Rea, 2007; Fukuhara et al., 1994). These genes are also transcribed in *Culex* mosquito testes as shown by qPCR and these genes could potentially be involved in circadian rhythms moving sperm from the testes to the seminal vesicles. In order to further study circadian rhythms in *Culex* mosquitoes, I performed qPCR to determine if *npyr*, *ssr* and *5-htr* are expressed in the testes. All three of these genes are expressed in the testes indicating a potential role in reproduction.

Introduction

Circadian rhythms in cellular activity occur in a roughly 24-hour cycle. They synchronize basic life functions in all living organisms (Giebultowicz, 1998). Circadian clocks, the rhythm generating mechanism, are not fully understood in any organism (Giebultowicz, 1998). In many animals, circadian rhythms are characterized by a self-sustained 24-hour period that persists under constant environmental conditions. This is why most circadian rhythms will continue to cycle under laboratory conditions without any time-associated cues (e.g. constant dark constant temperature). Often circadian rhythms are synchronized with (entrained to) external cues (Vitaterna et al., 2001), but they will continue in the absence of any exogenous time giving cues (zeitgebers). In other words, circadian rhythms are not driven by external cues but are aligned with them. In *Culex pipiens pallens*, a mating swarm, consisting of male mosquitoes, forms twice a day in the morning and in the evening (Chiba et al., 1982). This observation suggests that the preparation of sperm and mating behavior must be synchronized, which could potentially be controlled via circadian rhythms.

There is evidence for circadian rhythms controlling sperm movement from testes to seminal vesicles in other insects, specifically Lepidopterans (Giebultowicz et al., 1988; Giebultowicz et al., 1992; Bebas et al., 2001). In this case, sperm bundles are released from the testes into the upper vas deferens (UVD) and then to the seminal vesicles where the sperm are stored until mating. This movement is not a continuous process but rather a two-step rhythm entrained by the light-dark cycle. In the evening, sperm bundles are released from the testes to the UVD where they remain overnight. The sperm are then

transferred from the UVD to the seminal vesicles in the morning. This pattern of sperm release shows characteristics of a circadian rhythm in *Ephestia kuehniella* (Rieman et al., 1974; Thorson and Riemann, 1977) and in other Lepidopteran species (LaChance et al., 1977; Giebultowicz et al., 1988). In *Lymantria dispar*, the rhythmic release of sperm is maintained even in cultured male reproductive tracts (Giebultowicz et al., 1988), suggesting that the reproductive tract contains a circadian pacemaker in the absence of the central nervous system. This periodicity is important for successful reproduction in order to ensure that sperm is ready to be transferred to the female during times when they mate.

Circadian rhythms are controlled by a collection of clock genes. Circadian phases are controlled not only by the regulation of circadian clock genes but neuropeptide Y receptor (NPYR), serotonin receptor (5-HTR) and somatostatin receptor (SSR) are, also, involved in circadian rhythm regulation (Kennaway, 2005). NPYR and 5HTR provide inhibitory modulation of glutamatergic afferents (Marchant et al., 1997) and act on the suprachiasmatic nucleus to likely mediate circadian rhythm entrainment (Marchant et al., 1997). 5-HT_{2C} receptor agonists can mimic the effect of light pulses on circadian rhythms (Kennaway and Moyer, 1998), while NPY injection to the suprachiasmatic nucleus can shift the phase of hamsters (Albers and Ferris, 1984). Depleting somatostatin receptors causes phase advances of the rhythmic cycle of rat locomotor activity (Fukuhara et al., 1994). In rats, 5-HT_{2C} receptor mRNA demonstrates circadian rhythmicity of expression (Holmes et al., 1995) while in hamsters, 5-HT_{2A} and 5-HT_7 receptor mRNAs are photoperiodically regulated (Nilaweera et al., 2009).

Based on previous studies in other insect systems, and by my finding that sperm movements in the seminal vesicles are controlled by muscular contractions, I propose that sperm movement from the testes through the vas deferens to the seminal vesicles is controlled by circadian rhythms. In other organisms, circadian rhythms have been regulated via 5-HTR, NPYR and SSR. In this chapter, I show that *5-htr*, *npyr* and *ssr* are transcribed in the *Culex* testis, suggesting that they have a role in male reproductive processes. Expression of these transcripts in the testes, in combination with evidence in other organisms that they help modulate circadian rhythms, along with evidence in insects that circadian rhythms are involved in sperm transit from the testes, suggests a potential role for these genes in regulating movement of sperm through the testes via circadian rhythms.

Materials and Methods

RNA extraction

RNA was extracted from whole females as a positive control sample, and from male testes as the experimental sample. Total RNA was extracted using the TRIzol reagent according to the manufacturer's instructions. Contaminating DNA was degraded by treating each sample with RNAse-free DNase (Zymo Research) according to the instruction manual. Total RNA was quantified by optical density using the A260/280 ratio and the quality was evaluated by gel electrophoresis.

cDNA synthesis

One μg of total RNA was reverse transcribed using a Life Technologies High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's recommendations. Prior to use in qPCR cDNA was diluted 1:100 with RNAse/DNAse free-H_2O.

Primer Design

PrimerBlast and NetPrimer were used to design and analyze primer sequences for *5htr*, *npyr*, *ssr* and *ccap*r. PrimerBlast was used to design the primers and NetPrimer was used to predict the formation of primer dimers. Primers for each sequence were then ordered from Integrated DNA Technologies (Table 1).

Quantitative PCR with SYBR green

Each PCR reaction also included a reverse transcription negative control (RNA without reverse transcriptase treatment) to confirm the absence of genomic DNA and a non-template negative control to check for primer-dimer formation and a female cDNA positive control. Each reaction consisted of 20 µl containing 2 µl of cDNA and 50-200 nmol of each primer with SYBR green master mix (BioRad, Foster City, CA). Real time qPCR was run on a CFX96 or CFX96 Connect (Biorad) with the iQ5 Optical System software (Version 2.1) under the following cycling conditions: 40 cycles of 95 °C for 3 minutes, 95 °C for 10 seconds, and 58 °C for 30 seconds. The fluorescence was continually measured during PCR and one segment of product melting (65 °C with a 0.5 °C increase every 5 seconds up to 95 °C). Melting curves were constructed for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimers. All samples were amplified in triplicate and amplified products were normalized to actin. To analyze the data, I subtracted the average cycle threshold (C_T) from the number of cycles (40) for each gene including actin and then I normalized the expression of each gene to the value I calculated for actin.

Results

Transcription of Genes Potentially Involved in Regulating Circadian Rhythms

A DNA binding dye commonly used for qPCR, SYBR Green Supermix, was used to quantify the number of amplicons produced after each PCR cycle. SYBR Green binds DNA non-specifically and fluoresces when bound to the DNA major groove. The normal process of PCR involves denaturation, annealing and extension. SYBR Green binds and fluoresces during the annealing and extension step. At the completion of the annealing and extension step, the level of fluorescence is measured in order to quantify the amount of dsDNA (Figure 1). If there is more DNA present, the qPCR machine will register fluorescence sooner allowing expression levels to be determined based on the cycle at which fluorescence is first detected.

SYBR Green only fluoresces when bound to dsDNA and not in the presence of ssDNA or when free in solution. Once the thermal cycler finishes the qPCR amplification curves, a melt curve is produced. The thermal cycler starts at 65^{o}C degrees and is rasied in increments of 0.5^{o}C. The thermal cycler continues to measure fluorescence as the temperature increases. Since the intercalating dye (SYBR Green) only binds when the DNA is double stranded, the dye will dissociate and fluorescence will decrease as the dsDNA denatures. The slope change of the curve is then plotted as a function of temperature to generate the melting curve (Figure 2).

qPCR analysis demonstrated that *npyr*, *ssr* and *5-htr* were all transcribed in the testes and in the whole female; however, *ccapr* is not transcribed in the testes but is transcribed in the female (Figure 1 and 3). The transcription of *npyr*, *ssr* and *5-htr* in the

testes suggests that these three genes and their protein products may play a role in reproduction and perhaps delivery of sperm from the testis to the seminal vesicles. SSR, NPYR and 5-HTR are involved in circadian rhythm regulation in other systems and may serve similar functions in the testes of *Culex* mosquitoes.

Discussion

These results are preliminary, but, in conjunction with the literature, suggest a possible role for NPYR, SSR and 5-HTR in regulating movement of sperm from the testes, through the vas deferens to the seminal vesicles via circadian rhythms. In mammalian circadian rhythms, serotonin acts as a neurotransmitter that triggers or inhibits (light-induced) phase shifts, and controls sleep cycles (Bezerra de Pontes et al., 2010). Phase shifts are a change in the phase of a rhythm that is measured by a change in timing of a reference point (for example: nocturnal rise in melatonin) and can result in a phase delay where the reference point occurs later (peak levels are reached later) or phase advances where the reference point occurs earlier (peak levels are reached earlier). Microinjecting NPY into the mammalian SCN causes phase advances during the subjective day suggesting NPY is involved in modulating rhythmicity (Moore et al., 1984; Albers et al., 1984). NPY has been strongly implicated in photic and non-photic resetting of circadian clocks (Albers and Ferris, 1984; Biello et al., 1994; Marchant et al., 1997; Yannielli and Harrington, 2004). Treatment with somatostatin changes the phase of the circadian rhythm and somatostatin is expressed rhythmically even under constant darkness suggesting that somatostatin is involved in either generating or mediating the generated circadian rhythms (Hamada et al., 1993).

Previous studies suggest that circadian rhythms are involved in moving sperm from the testes to the seminal vesicles and that NPYR, SSR and 5-HTR are involved in regulating circadian phases. Based on these observations, I performed qPCR assays and determined that *npyr*, *ssr* and *5-htr* are all expressed at detectable levels in the testis

suggesting that further studies investigating circadian rhythms and their regulators are warranted.

In order to further investigate the role of circadian rhythms in *Culex* sperm movement and particularly, how NPYR, SSR and 5-HTR are involved in the regulation of circadian rhythms, we could start by determining if the circadian rhythm genes are transcribed in the testes (Figure 2). Next, a time course would be performed with *Culex* testes removed every four hours and RNA extracted and then subsequently converted to cDNA. The cDNA produced could then be used for qPCR to determine if *5-htr*, *sstr*, *npyr* and circadian genes are transcribed in a rhythmic fashion in the testes (Figure 4). If the genes are transcribed in a rhythmic fashion in the testes, one could then knockdown *5-htr*, *ssr* or *npyr* in a testes specific context and determine if the rhythmic nature of the circadian genes expression is disrupted (Figure 4). This would inform us of whether or not NPYR, SSR and 5-HTR are involved in regulating the circadian genes. In addition, the opposite and complementary approach could also be done, namely, we can knockdown the circadian genes and determine if the rhythmic nature of *5-htr, ssr* or *npyr* is disrupted (Figure 4). This would establish whether or not circadian genes are involved in regulating *5-htr*, *ssr* or *npyr*.

If we confirmed that *5-htr*, *ssr* and *npyr* genes were transcribed in a rhythmic fashion in the testes and they regulate each other's expression, we could then explore their physiological role in mosquito reproduction. There is a growing body of evidence that circadian rhythm and reproduction is interconnected (Boden and Kennaway, 2005). In moths there is a growing body of evidence that circadian rhythms control the

movement of sperm from the testes to the seminal vesicles (Thorson and Riemann, 1977; Riemann et al., 1974; Giebultowitcz et al., 1988; Bebas et al., 2001; Giebultowitcz et al., 1988). Based on this, I would explore the role of circadian rhythms in *Culex* sperm movement. To do this we could keep males in a light:dark cycle of 16:8 (Based on Giebultowicz et al., 1996) and dissect males every four hours and determine where the sperm is located at each time point (Figure 5). This will help us to determine if the sperm movement from the testes is rhythmic. Once we have done this we could knockdown *5-htr*, *ssr*, *npyr* and circadian rhythm genes and repeat the above time course to determine if knocking down any of these genes changes the rhythmicity of sperm movement from the testes (Figure 5).

Mosquitoes are vectors of several diseases that cause high mortality and morbidity. In fact, mosquitoes as a whole are responsible for more deaths than any other animal species on earth (WHO, 2004). The primary vector for West Nile Virus is the *Culex* mosquito, which can be effectively controlled using pyrethroid pesticides. However, many populations of *Culex* have developed insecticide resistance to these pesticides (Ranson et al., 2011; Asidi et al., 2012; Wondji et al., 2012; Corbel et al., 2007; Li and Liu, 2010) leading to worldwide problem when attempting to control *Culex* populations. Since *Culex* populations are developing resistance to conventional pesticides it is necessary to develop new-targeted methods to control them. Controlling fertilization in mosquitoes can be used to control populations and understanding the role of circadian rhythms in mosquito reproduction could assist in developing new methods to control the mosquito populations and therefore reduce disease transmission.

References

Albers and Ferris. (1984). Neuropeptide Y: role in light–dark cycle entrainment of hamster circadian rhythms. *Neurosci. Lett.* 50:163–168

Albers H.E., Ferris C.F., Leeman S.E. and Goldman B.D. (1984). Avian pancreatic polypeptide phase shifts hamster circadian rhythms when microinjected into the suprachiasmatic region. *Science*. 223: 833-835.

Alvarez J.D., Chen D., Storer E. and Sehgal A. (2003). Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. *Biol. Reprod.* 69:81–91.

Asidi A., N'Guessan R., Curtis C. and Rowland M. (2012). Loss of household protection from use of insecticide-treated netsagainst pyrethroid-resistant mosquitoes, *Benin. Emerg. Infect. Dis.*18: 1101–1106.

Bebas P., Cymborowski B., Giebultowicz J.M. (2001). Circadian rhythm of sperm release in males of the cotton leafworm *Spondoptera littoralis*: *in vivo* and in vitro study. *J. Insect Physiol.* 47: 859-866.

Bezerra de Pontes R.C.G.J., Engelberth E., da Silva Nascimento J.C., Cavalcante, M.S.M., de Oliveira Costa L. Pinato C.A., Barbosa de Toledo J., de Souza Cavalcante. (2010). Serotonin and circadian rhythms. *Psychol. Neurosci*. 3: 217–228

Biello S.M. and Mrosovsky N. (1994). Blocking the phase-shifting effect of neuropeptide Y with light. *Proc. R. Soc. Lond. B Biol. Sci.* 259: 179–187.

Boden M.J. and Kennaway D.J. (2005). Circadian rhythms and reproduction. *Reproduction.* 132:379–392.

Corbel V., N'Guessan R., Brengues F., Chandre F., Djogbenou L., Martin T., Akogbeto M., Hougard J.M. and Rowland M. (2007). Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica.* 101: 207–216.

Chiba, Kubota and Nakamura. (1982). Differential effects of temperature upon evening and morning peaks in the circadian activity of mosquitoes, *Culex pipiens pallens* and *C. pipiens molestus. J. interdiscipl. Cycle Res.* 13 (1): 55-60.

Fukuhara C., Hamada T., Shibata S., Watanabe S., Aoki K., Inouye S.I. (1994). Phase advances of circadian rhythms in somatostatin depleted rats: effects of cysteamine on rhythms of locomotor activity and electrical discharge of the suprachiasmatic nucleus. *J Comp. Physiol.* 175:677–685.

Giebultowicz J.M., Bell R.A. and Imberski R.B. (1988). Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar*. *J. Insect Physiol*. 34:527-532

Giebultowicz J.M., Joy J.E. and Riemann J.G. Circadian rhythm of sperm release from the testis in moths: protein changes during development of the sperm release system. In Advances in Regulation of Insect Reproduction (Edited by: Benetova B, Gelbic I, Soldan T) Ceske Budejovice: Czech Acad Sci 1992, 91-95

Hamada T., Shibata S., Tsuneyoshi A., Tominaga K. and Watanabe S. (1993). Effect of somatostatin on the circadian rhythms of firing and 2-deoxyglucose uptake in the rat suprachiasmatic slices. *Am. J. Physiol.* 265:R1199-R1204

Holmes M.C., French K.L. and Seckl J.R. (1995). Modulation of serotonin and corticosteroid receptor gene expression in the rat hippocampus with circadian rhythm and stress. *Mol. Brain Res.* 28:186–192.

Kennaway D.J. (2005). The role of circadian rhythmicity in reproduction. *Hum. Reprod. Update.* 11:91–101.

Kennaway D.J. and Moyer R.W. (1998). Serotonin 5-HT2c agonists mimic the effect of light pulses on circadian rhythms. *Brain Res*. 806 (2): 257- 270

LaChance L.E., Richard R.D. and Ruud R.L. (1977). Movement of eupyrene sperm bundles from the testes and storage in the ductus ejaculatoris duplex of the male pink bollworm: effects of age, strain, irradiation and light. *Ann. Ent. Soc. Am*. 70:647-652

Li T. and Liu N. (2010). Inheritance of permethrin resistance in *Culex quinquefasciatus*. *J. Med. Entomol.* 47, 1127–1134.

Livak K. and Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 ΔΔCT method. Methods. 25: 402–408.

Marchant, Watson and Mistlberger. (1997). Both neuropeptide Y and serotonin are necessary for entrainment of circadian rhythms in mice by daily treadmill running schedules. *J. Neurosci.* 17: 7974–7987.

Moore R.Y., Gustafson E.L. and Card J.P. (1984). Identical immunoreactivity of afferents to the rat suprachiasmatic nucleus with antisera against avian pancreatic polypeptide, molluscan cardioexcitatory peptide and neuropeptide Y. *Cell and Tissue Research*. 236: 41-46.

Nilaweera K.N., Archer Z.A., Campbell G., Mayer C.D., Balik A., Ross A.W., Mercer

J.G., Ebling F.J., Morgan P.J. and Barrett P. (2009). Photoperiod regulates genes encoding melanocortin 3 and serotonin receptors and secretogranins in the dorsomedial posterior arcuate of the Siberian hamster. *J. Neuroendocrinol.* 21: 123–131.

Ranson H., N'guessan R., Lines J., Moiroux N., Nkuni Z. and Corbel V. (2011). Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol.* 27: 91–98.

Riemann J.G., Thorson B.J. and Ruud R.L. (1974). Daily cycle of release of sperm from the testes Mediterranean flour moth. *J. Insect Physiol.* 20:195-207

Thorson B.J. and Riemann J. (1977) Abdominally entrained periodicities of testis and vas deferens activity in the Mediterranean flour moth. *J Insect Physiol*. 23:1189-1197

WHO. *The World Health Report 2004: Changing History* (WHO, 2004).

Yannielli P.C. and Harrington M.E. (2004). Let there be "more" light: enhancement of light actions on the circadian system through non-photic pathways. *Prog. Neurobiol.* 74: 59–76

Figures and Tables

Table 5.1. List of primer sequences used for this study.

Primer	**Sequence**	**Base Pairs**	**Gene ID**
Actin F	GCCCAATCCAAGCGAGGTAT	271	CpipJ_CPIJ012090-RA
Actin R	TACGACCGGAAGCATACAGC	271	
NPY F	TTCTTCATCGCCCACCTCAC	198	CpipJ_CPIJ013069.3
NPY R	GGTTTCCTGGACAGTGTCGT	198	
CCAP F	GGGCATCATTCCAAAGGCGA	158	CpipJ_CPIJ006269.3
CCAP R	TGAAGGTCGCTACGGCGATA	158	
5HT F	GCCATTTTCGCCAACTCGTC	311	CpipJ_CPIJ016039.3
5HT R	ACCGCCACACATACTAGCAC	311	
SSR F	CACATCTCGTCGCCCAAGTA	272	CpipJ_CPIJ011191.3
SSR R	CGCAGTTTCCGTATCACCAG	272	

Figure 5.1A. Amplification curve for *ssr*. The x-axis shows the cycle number and the y-axis shows the relative fluorescence units. The graph shows how many cycles it took for fluorescence to be detected by the qPCR machine. Each line represents line represents a different biological or technical sample.

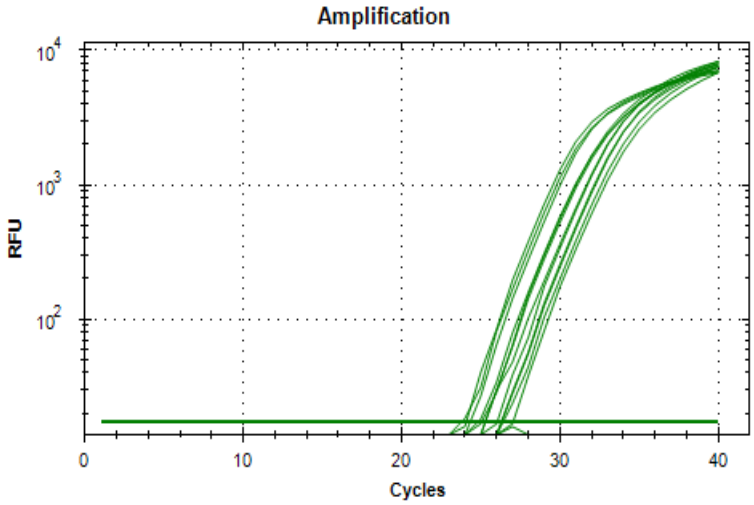


Figure 5.1B. Amplification curve for *npyr*. The x-axis shows the cycle number and the y-axis shows the relative fluorescence units. The graph shows how many cycles it took for fluorescence to be detected by the qPCR machine. Each line represents line represents a different biological or technical sample.

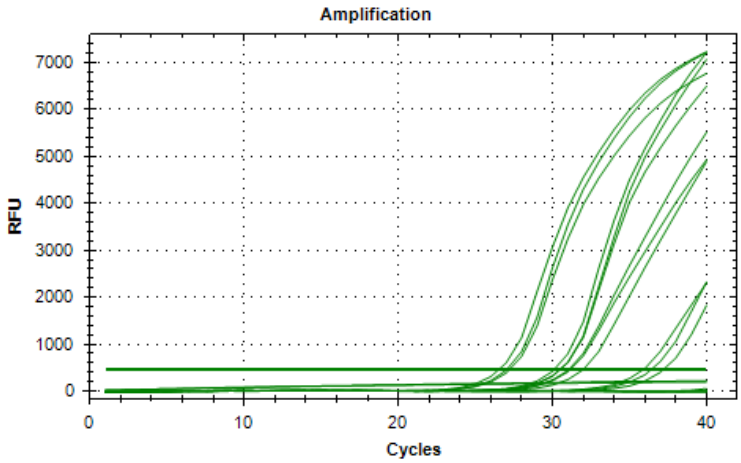


Figure 5.1C. Amplification curve for *ccapr*. The x-axis shows the cycle number and the y-axis shows the relative fluorescence units. The graph shows how many cycles it took for fluorescence to be detected by the qPCR machine. Each line represents line represents a different biological or technical sample.

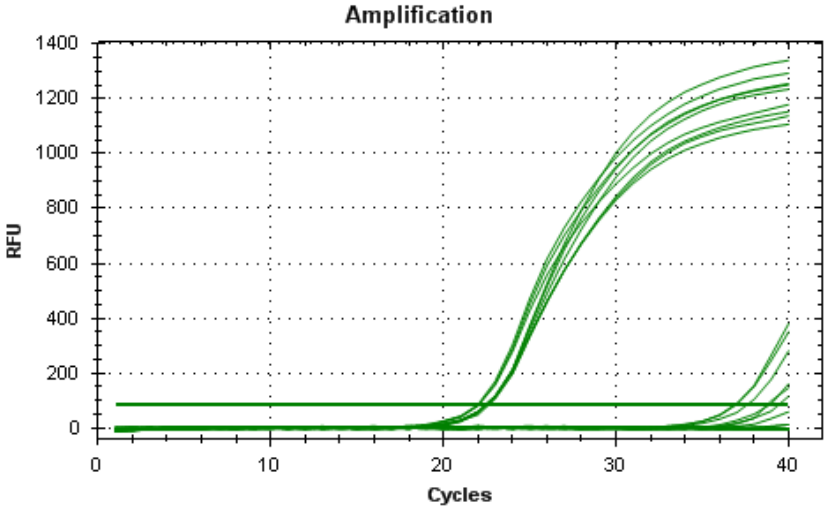


Figure 5.1D. Amplification curve for *5htr*. The x-axis shows the cycle number and the y-axis shows the relative fluorescence units. The graph shows how many cycles it took for fluorescence to be detected by the qPCR machine.

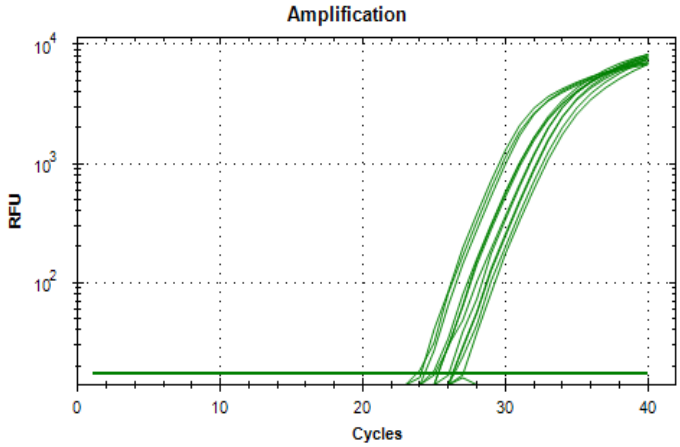


Figure 5.2A. Melt curve for *ssr*.

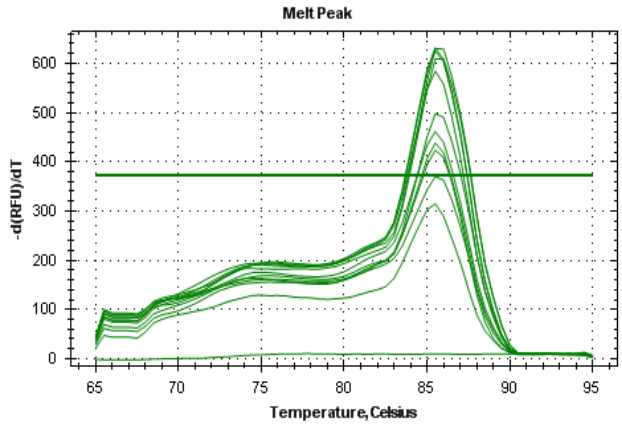


Figure 5.2B. Melt curve for *npyr*.

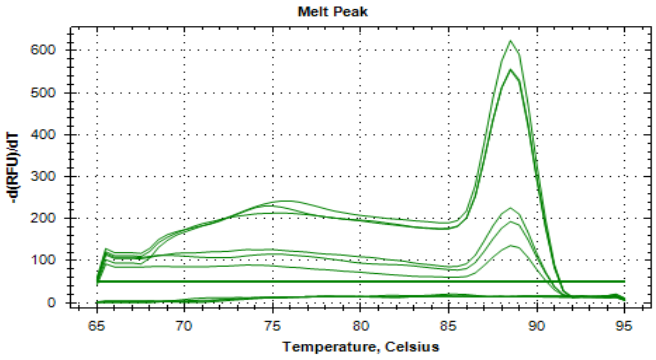


Figure 5.2C. Melt curve for *ccapr*.

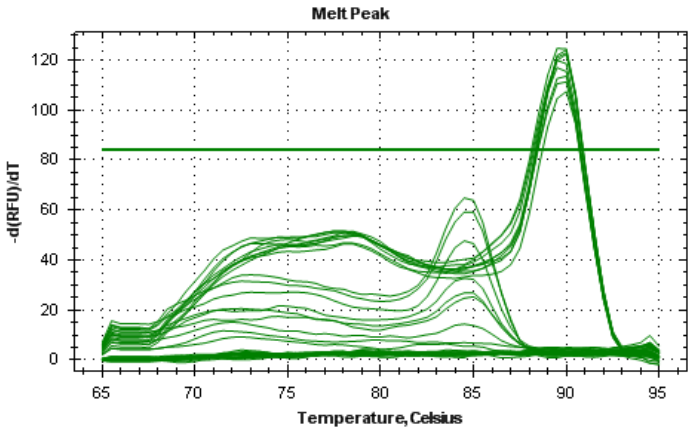


Figure 5.2D. Melt curve for *5htr*.

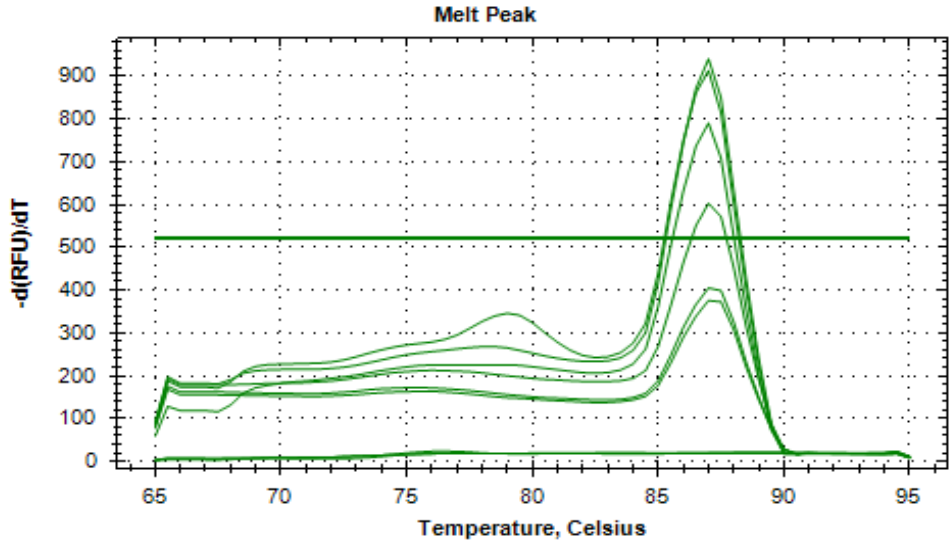


Figure 5.3. qPCRs were performed using testes tissue (test sample) and whole females (control) to test for the presence of four transcripts. Three of four genes were expressed in the testes. To analyze the data, I subtracted the average cycle threshold (C_T) form the number of cycles (40) for each gene including actin and then I normalized the expression of each gene to the value I calculated for actin.

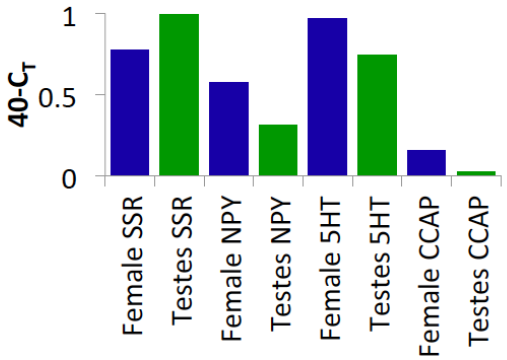


Figure 5.4. Outline of future experiments.

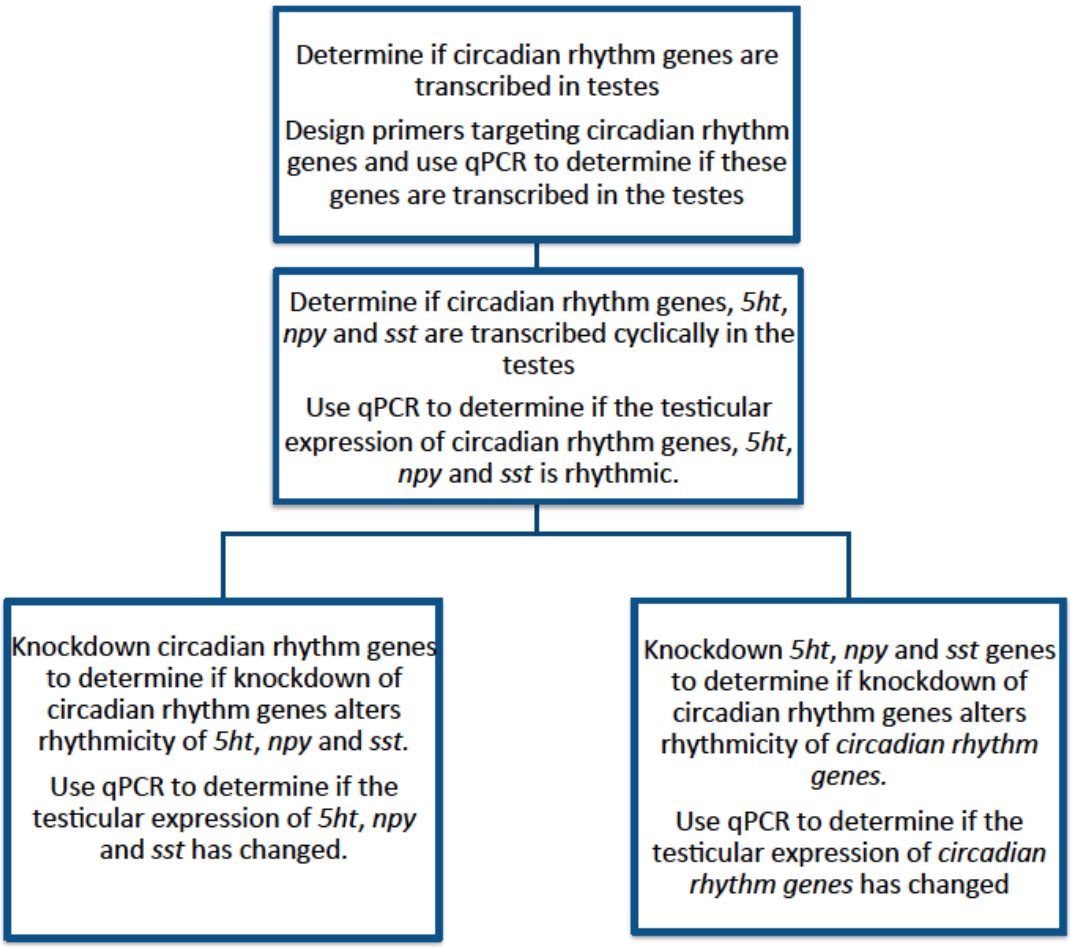
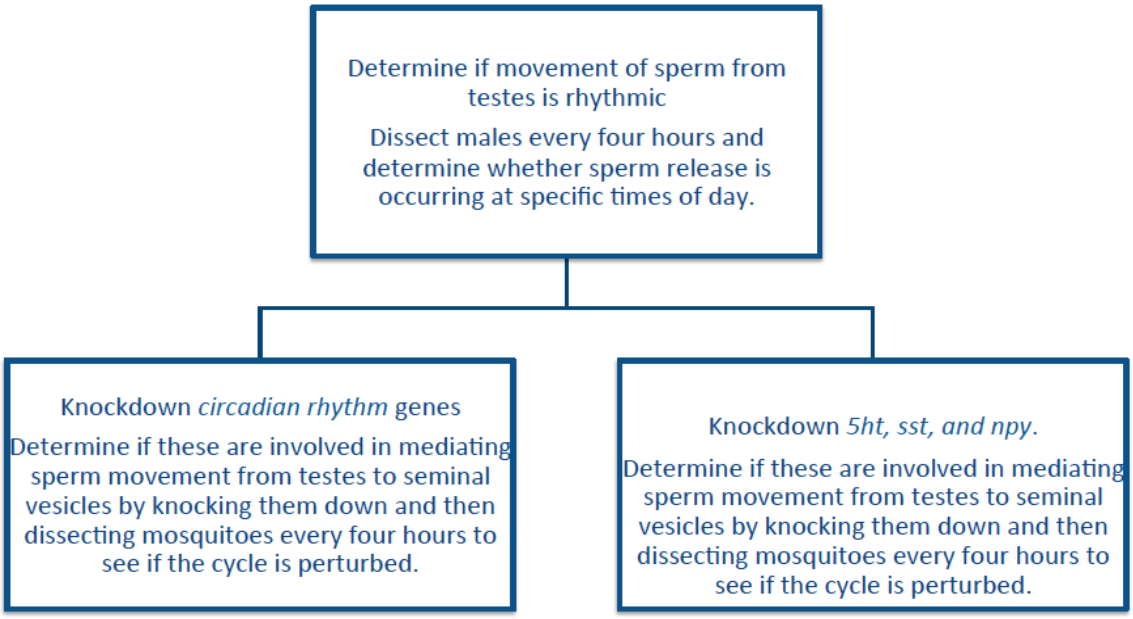


Figure 5.5. Outline of future experiments.



CHAPTER 6

Discussion

Production of a mature sperm from a stem cell is a complex process that occurs in the testes but the ultimate function of the sperm is not realized until the sperm interacts with the egg. During its journey, sperm in internally fertilizing species undergo several highly regulated developmental stages as they make their way through the male and female reproductive tract. Very little is known about movement and modification of sperm from the male testes through the female reproductive tract. The overall goal of this dissertation has been to describe the movement of sperm from the testes to the egg in water striders and *Culex* mosquitoes. I followed the life history of the sperm from movement out of the testes, through the seminal vesicles and eventually to the egg where the sperm interacts with the egg. Microscopic, biochemical and molecular methodologies were used to explore the life history of the sperm. In this final chapter, I discuss sperm movement from testes to seminal vesicles, motility regulation and, lastly, sperm-egg interactions.

Movement from testes to seminal vesicles

In mammals, the main function of the testis is two fold: steroidogenesis in the Leydig cells and spermatogenesis in the Sertoli cells of the seminiferous tubules. Spermatogenesis is the sum of the entire transformative process from the undifferentiated male germ cell, spermatogonia, to spermatozoa while maintaining spermatogonial numbers (Courot et al., 1970; Ortovant et al., 1977). This process involves mitotic divisions of spermatogonia to form spermatocytes, which then undergo meiosis to produce spermatids that subsequently differentiate into spermatozoa. When sperm leave

the testis they are infertile and incapable of fertilizing an egg. In order for a sperm to become motile and fertilization competent, it must leave the testis and enter the epididymis, a highly convoluted duct, which facilitates sperm maturation and serves as the sperm storage organ (Cooper et al., 2004; Jocelyn et al., 1972; Robaire et al., 2006; Robaire et al., 2002; Turner and de Graaf, 2008). Sperm move along this duct aided by smooth muscle contractions and pressure from fluid/sperm entering the epididymis (Cooper et al., 2004; Jocelyn et al., 1972; Robaire et al., 2006; Robaire et al., 2002; Turner and de Graaf, 2008). Sperm are then moved to the ductus deferens, which carries them to the ejaculatory duct. In the ductus deferens, sperm are still immotile and transported via peristalsis (Kuriyama et al., 1998; Feletou and Vanhoutte, 2000; Steers et al., 1994). The walls of the ductus deferens are lined with smooth muscle, which contracts to move sperm along its length (Kuriyama et al*.,* 1998; Feletou and Vanhoutte, 2000; Steers et al., 1994). It can then be stored in the epididymis for several months until sperm are delivered to the female through ejaculation (Kuriyama et al*.,* 1998; Feletou and Vanhoutte, 2000; Steers et al., 1994).

Like mammals, insect sperm are produced in the testes and sperm production begins with a series of mitotic divisions followed by meiosis, and subsequently by nuclear condensation, acrosome formation, and flagellar development (Fernandes et al., 2001). However, there are aspects of spermatogenesis that are unique to insects. One aspect of spermatogenesis that is unique to insects is that the gonialblast undergoes multiple rounds of replication before initiating meiosis (Fuller, 1998). The number of rounds of mitosis that occur prior to meiosis is insect specific. Cytoplasmic bridges that

connect the cells from the gonialblast are formed due to incomplete cytokinesis during both mitosis and meiosis. These cells remain interconnected and will develop synchronously during spermiogenesis (Dias et al., 2012). Insects also form a nebenkern, which is a large mitochondrial structure formed by aggregation and fusion of multiple mitochondria. The division of the nebenkern forms two separate bodies, these are called mitochondrial derivatives and they extend the length of the flagellum (Fabian and Brill, 2012).

Following spermiogenesis, insect sperm move from the testes through the vas deferens (ductus deferens) and subsequently to the seminal vesicles where they are stored. Compared to mammals, very little is known about sperm transport through the male reproductive tract in insects. Circadian rhythms are known to play a role in various aspects of insect reproduction including courtship, copulation, egg deposition, oogenesis and spermatogenesis (Tobback et al., 2011). Regulation of movement of sperm via the peripheral circadian system has been described in many moth species. The movement of sperm from the testes into the upper vas deferens only occurs a few hours each day independent of the central clock (Riemann et al., 1974; Giebultowicz et al., 1988; Giebultowicz et al., 1998; Bebas et al., 2001). The period gene is rhythmically expressed in the moth testis-upper vas deferens complex (Gvakharia et al., 2000). Constant light disrupts the circadian rhythm and results in sterility (Giebultowicz, 1990; Bebas et al., 1999; Bebas et al., 2002a, b). In females, knockdown of clock genes results in fewer eggs developing suggesting that clock genes are also involved in regulating oogenesis as well (Tobback et al., 2011). Beyond the results implicating circadian rhythms, little is known

regarding how the sperm bundles are moved out of the testes. My research shows that three genes that modulate circadian rhythms in mammals, *npyr*, *ssr* and *5-htr*, (Kennaway, 2005) are transcribed in the *Culex* testes. Thus, it is possible that circadian rhythms are involved in sperm movement from the testes in *Culex* as well. Thus, it will be important to investigate the role of circadian rhythms in mosquito sperm movement. I would be interested in determining if the process of sperm movement from the testes is conserved in multiple orders of insects. If we confirm that the circadian rhythm, *5-ht*, *srt* and *npyr* genes are transcribed in a rhythmic fashion in the testes and regulate each other's expression we can then explore their physiological role in mosquito reproduction.

After sperm exit the testes and pass through the vas deferens, they enter the seminal vesicles. In earwigs (*Euborellia annulipes*), accessory gland, seminal vesicle and ejaculatory duct muscles undergo contractions that are hypothesized to move the sperm and seminal fluid from the accessory gland, seminal vesicle and ejaculatory duct into the female (Rankin et al., 2009). In crickets (*Gryllus bimaculatus*), accessory gland contractions (Kimura et al., 1989) are hypothesized to push the seminal fluid into the ejaculatory duct. In both earwigs and crickets, proctolin has been found to stimulate contractions (Rankin et al., 2009; Kimura et al., 1989) suggesting proctolinergic activation of male reproductive tissues. I found that a PDE suppressor, which causes an increase in cyclic nucleotides, induces muscle contractions. Proctolin mediates rhythmic muscle contractions by increasing the levels of a specific cyclic nucleotide, cAMP (Evans, 1984) in a calcium dependent manner (Wilcox and Lange, 1995). These observations are consistent with my finding that suppressing phosphodiesterases and,

thereby, increasing cyclic nucleotide levels leads to muscle contractions in the *Culex* male reproductive tract, but only in the presence of calcium. One possibility is that a PKA pathway is activated by proctolin binding a receptor in the reproductive tissue of male *Culex*. To test this, a PKA specific activator and proctolin still need to be tested. In *Culex*, I also found that muscle contractions are stimulated by addition of nicotine and acetylcholine suggesting a nicotinic acetylcholine receptor is also involved in activation of muscle contractions.

Motility regulation

Another step in sperm maturation is the activation of sperm motility, which is often regulated via extracellular cues that include changes in either pH or viscosity (Yoshida et al., 2008). However, in insects, it is not uncommon for sperm motility initiation to require proteases such as trypsin, which has been observed in holometabolous and hemimetabolous insects (Osanai and Baccetti, 1993; Friedländer et al., 2001). Previous research from the Cardullo laboratory has shown that in both *A. remigis* (Hemiptera, hemimetabola) and *Culex quinquefasciatus* (Diptera, holometabola) sperm the addition of trypsin is sufficient to initiate vigorous motility (Miyata et al., 2012; Thaler et al., 2014). In addition, *Culex quinquefasciatus* seminal vesicle sperm is spontaneously initiated when exposed to the endogenous initiator present in the accessory glands (Thaler et al., 2014). However, in the absence of calcium, sperm did not activate motility (Thaler et al., 2014). In addition, activated sperm become immotile when EGTA is added (Thaler et al., 2014). Addition of a protease inhibitor, aprotinin, blocked the

transition from Wave A to Wave B (Thaler et al., 2014). Interestingly, low calcium and increased phosphorylation results in backwards motility (Thaler et al., 2014). Together, these results indicate that trypsin activates sperm motility and calcium is important for sperm activation. Subsequent pharmacological studies revealed that trypsin cleavage of a protein on the sperm membrane likely causes the activation of a MAPK pathway (Thaler et al., 2014). In *Culex* and *A. remigis*, trypsin activation of sperm motility by the MAPK-pathway presumably results in phosphorylation of axonemal proteins to stimulate motility.

My studies have expanded on this by demonstrating that the accessory glands have trypsin-like activity and identifying the trypsin-like proteases that are present in the accessory glands. It is likely that these serine proteases are involved in activating the sperm motility as other studies have demonstrated that trypsin or an accessory gland extract is capable of activating sperm motility (Miyata et al., 2012; Thaler et al., 2014). It is likely that when sperm mix with the accessory gland fluid during ejaculation they are activated. Serine proteases that were identified in the accessory gland proteome might be involved in maintaining sperm quiescence until the sperm reach the female. This level of regulation would be necessary to maintain sperm quiescence and minimize energy utilization prior to entering the female reproductive tract.

In addition to serine proteases, pH might also maintain sperm quiescence until the sperm and accessory gland fluid reach the female, which is consistent with my finding that the trypsin-like protein acts within a very narrow pH range. It is possible that the reproductive tract of a receptive and fertile female maintains a narrow pH range of

approximately 8 within a specific region of the reproductive tract to ensure that sperm activation occurs at the optimal time and place for successful fertilization. This would be especially important if the accessory gland fluid and sperm are transferred simultaneously in the female reproductive tract.

The presence of a trypsin-like protease in the male accessory glands and the ability of accessory gland fluid to activate sperm motility support the hypothesis that a PAR2-like protein is responsible for activation of sperm motility (Miyata et al., 2012; Thaler et al., 2014). PARs are GPCRs that are activated through the cleavage of a specific protease (Cottrell et al., 2003). PAR2 is cleaved by trypsin between the arginine and serine residues of an RSLIGKV (human) or an RSLIGRL (mouse) sequence (Cottrell et al., 2003) and receptor activation stimulates MAPK signaling via a β-arrestin-dependent mechanism (Cottrell et al., 2003; Ramachandran et al., 2009).

The only PAR2 protein currently known to exist in insects is present on the *A. remigis* sperm flagellum detected by an anti-PAR2 antibody directed at the N-terminus of the mouse PAR2 (Thaler et al., 2014). This result provides further evidence of a PAR2-like protein being cleaved by trypsin to stimulate activation. This particular method of sperm motility activation is, to date, unique to insects. To detect a PAR2-like protein, I submitted sperm samples to a mass spectrometry core facility. The analysis revealed one GPCR, OR7/ORCO (Figure 1). ORs are expressed in *A. gambiae* sperm where they are likely involved in sperm activation and orientation (Pitts et al., 2014) indicating that ORCO is a potential candidate for the PAR2-like protein involved in sperm activation. Although, OR7 is present in the sperm proteome it has a reverse topology that has an

intracellular N-terminus and an extracellular C-terminus. Within the extracellular C-terminus, OR7 has potential RSLIGRL cleavage sites (Figure 1). To determine if OR7 is transcribed in the testes I performed qPCR and found that ORCO is transcribed in the male testes as well as in the whole body of the female (Figure 2). To further support a role for ORCO as the PAR2-like protein involved in sperm activation, I tested the ORCO inhibitors, 5-(N,N –hexamethylene)amiloride (blocks sodium-hydrogen antiporter) and KB-R7943 (blocks sodium calcium exchange). Both of these pharmacological agents inhibited sperm motility within 10 minutes after activation (Table 1). In order to further assess ORCOs role in sperm motility, ORCO can be knocked down and sperm subsequently assessed for changes in ability to activate motility. In order to do this, I would use the CRISPR-cas9 system. Primers for gRNA will be designed using a gRNA design tool available online and *E.* coli would be used to produce gRNA. MaxiPrep would be used to isolate the plasmid DNA. For cas9 to bind, the genomic target sequence must have a protospacer adjacent motif (PAM) immediately following the target sequence. Guide RNAs (gRNA) then form a complex, which localizes the cas9 to the target sequence resulting in cas9 cutting resulting in a double strand break (DSB) of the DNA. The DSB is repaired leading to either gene disruption via Non Homologous End Joining (NHEJ) or gene editing via homology directed repair (HDR). I would use both types of gene disruption to study the function of potential PAR2-like proteins that I identify. I would specifically target the RSLIGRL sequence for either deletion or replacement. The constructs (gRNA, cas9 and donor plasmid necessary to replace RSLIGRL sequence) would be introduced into *Culex* mosquitoes by injection into the

embryo. I would then design primer pairs flanking the gRNA cut sites and primers specific for the sequence used to replace the HDR. I would use PCR to determine if the DNA has inserted correctly. Complementation studies would be performed in order to ensure that phenotypes assessed are from the mutation of the candidate *par2* gene. Once we have PAR2-like mutants, I would determine if the mutations affect sperm motility. To do this, I would treat mutant and non-mutant mosquito sperm with trypsin or accessory gland fluids. If mutant sperm is not activated by trypsin that indicates that the PAR2-like protein that has been mutated is involved in activating sperm motility.

Sperm-egg interaction

Once the gametes have moved from the testes to the seminal vesicles and subsequently ejaculated into the female, the sperm must find, recognize and contact the egg in a species-specific manner. Modeled in this way a specific receptor on the sperm plasma membrane binds to a specific ligand on the matrix surrounding the egg (e.g., the zona pellucida in mammals and other vertebrates or the vitelline envelope in echinoderms as well as other taxa or chorion in insects). In species that exhibit external fertilization, this level of recognition and binding prevents cross-fertilization between different species since the sperm receptor of one species is unable to recognize and bind the cognate ligand on the egg (Minor et al., 1991; Minor et al., 1993). In species with internal fertilization, a receptor-ligand interaction between the sperm and egg may be involved in helping to direct the sperm to the site of entry. Currently, only a few putative species-specific receptor-ligand interactions involved in fertilization have been explored and most of

these have been limited to deuterostome lineages (Vacquier and Payne, 1973; Glabe and Vacquier, 1978; Kamei and Glabe, 2003).

There is evidence in several organisms that the molecules involved in primary sperm-egg binding are carbohydrate-binding proteins (lectins) on the sperm surface that recognize complementary glycans on the egg surface (Downey and Lambert, 1994; Habermann and Sinowatz, 2011; Sinowatz et al., 1998; Focarelli et al., 2001). One hypothesis is that enzymes such as glycosidases and glycosyltransferases function as lectins may be involved in the primary recognition event between sperm and egg by not completing their enzymatic cycle (Mengerink and Vacquier, 2001; Sinowatz et al., 1998; Tian et al., 1997; Downey and Lambert, 1994; Matsumoto et al., 2002; Tulsiani et al., 1997; Tulsiani et al., 1989; Zitta, Werthheimer, and Miranda, 2006; Shur et al., 2006; Lopez et al., 1985; Lopez and Shur, 1987; Shur and Hall, 1982; Shur, 1988). In the case of a glycosidase, the glycosidase-sugar complex would form but be unable to cleave the appropriate carbohydrate thus leading to a situation in which the sugar ligand is bound to the active site of the enzyme. Further, if catalysis were to occur, the terminal sugar would be cleaved and the sperm would dissociate from the egg. However, the affinity for the glycan on the egg would need to be low enough to allow the sperm to detach from the matrix upon entry. In water strider sperm, the K_m for 4-NP-NAG was 0.093 ± 0.01 mM and the K_m for 4-NP-Man was 2.36 ± 0.19 mM, which is low affinity. Importantly, enzymes are extremely specific in the types of reactions that they can catalyze having either absolute, group, linkage or stereochemical specificity (Seager and Slabaugh, 2010). This high level specificity makes enzymes appropriate and likely candidates for receptor-

ligand interactions.

Examples of glycosidases being present on the sperm plasma membrane include mannosidases in mammalian sperm, fucosidases and N-acetylglucosaminidases in tunicates, and mannosidases, fucosidases and N-acetylglucosaminidases in flies (Tulsiani et al., 1997; Godknecht and Honegger, 1991; Matsumoto et al., 2002; Downey and Lambert, 1994; Perotti, 2001; Pasini et al., 1999; Intra et al., 2006; Intra et al., 2011; Cattaneo et al., 1997). In mammalian sperm, mannosidase has been hypothesized to be a receptor involved in sperm-egg recognition because it is an integral sperm plasma membrane protein and treatment of sperm with either D-mannose or an antibody raised against mannosidase elicit a dose-dependent inhibition of sperm–zona binding (Cornwall et al., 1991; Tulsiani, 2000; Tulsiani et al., 1989; Yoshida-Komiya et al., 1999; Pereira et al., 1998). Steric hindrance might affect results when it comes to antibodies inhibiting sperm-egg binding. It is possible that the large size of an antibody raised against mannosidase would unintentionally block the binding of nearby proteins or sugars suggesting that any membrane protein could be implicated using this technique. Therefore, studies involving antibodies should be confirmed using other methods. In the tunicate, *Ciona intestinalis*, a sperm fucosidase and egg fucose are thought to be involved in sperm egg binding (Rosati, 1985). The fibrillar tufts on the vitelline coat of *Ciona intestinalis* react to lectins that recognize fucose (Rosati and de Santis, 1978) and fucosidase is present on the sperm surface (Hoshi et al., 1983). Further, when eggs and sperm are suspended in seawater, sperm-egg binding is inhibited by the presence of 25-50 mM L-fucose but not 25-50 mM of other sugars (Rosati and De Santis, 1980) or the

presence of enzymatic substrates, pNP-**α**-L-fucose and pNP- **β**-L-fucose (Hoshi et al., 1983). In the tunicates *Ascidia nigra* and *Phallusia mamillata*, a sperm N-acetylglucosaminidase is thought to recognize egg N-acetylglucosamines (Rosati and Santis, 1978). For both *Ascidia nigra* and *Phallusia mamillata*, the conclusion that a sperm N-acetylglucosaminidase and an egg N-acetylglucosamine (NAG) are involved in sperm-egg recognition is based on binding competition assays with NAG-glycosides (Rosati, 1985; Rosati and de Santis, 1978). When eggs and sperm are suspended in seawater a high concentration of sugars might be necessary to inhibit sperm-egg binding because the free sugars would need to compete with the natural substrate, which may have the same composition of sugars but different structures. The inhibitor concentration used to get maximal binding inhibition depends on substrate (ligand) concentration, enzyme (receptor) concentration and the affinity of the inhibitor versus affinity of the substrate (Cer et al., 2009). In *Phallusia mamillata*, sperm have N-acetylglucosaminidase localized to the plasma membrane and the localization of the enzyme redistributes as a result of the acrosome reaction (Godknecht and Honegger, 1991). In order to confirm the results of inhibition studies, knockout studies are needed.

Surprisingly, in systems that utilize genetic knockouts, such as β-D-Gal-α1,3-Gal-galactosyltransferase in mice, the animals remained fertile despite being unable to produce oocytes with Gal-α1,3-Gal epitopes (Thall et al., 1997). Another knockout mouse that prevents the addition of N-acetylglucosamine and galactose also did not reduce fertilization (Williams et al., 2007). Additionally, sperm from β1,4-galactosyltransferase-null mice are still able to penetrate the egg (Lu and Shur, 1997).

These data could indicate that redundant mechanisms have evolved to provide a fail-safe. It is more likely that spem-egg binding is a result of coordinated interactions between multiple receptors and their ligands as opposed to a single interacting pair. Redundancy in the receptor/ligand pairs could also lead to sequential recognition events with varying affinity as is seen in mouse gamete interactions (Thaler and Cardullo, 1996; Thaler and Cardullo, 2002). The functional redundancy in the system is commensurate with the overall importance of the interaction (Clark and Dell, 2006).

In insects, little is known about the molecular basis of sperm-egg interactions. *Drosophila* sperm possess α-L-fucosidase, α-mannosidase and β-N-acetylhexosaminidase and the egg micropyle is coated with the respective sugars suggesting a possible role in the sperm-egg interaction (Intra et al., 2006; Intra et al., 2011; Perotti, 2001). The role of β-N-acetylhexosaminidase is further substantiated with the *Drosophila melanogaster Casanova* mutant. In the *Casanova* mutant, the sperm head lacks β-N-acetylhexosaminidase and the sperm is unable to penetrate the egg (Perotti, 2001). The presence of β-N-acetylhexosaminidase on the sperm head is conserved across the *Drosophila* genus. *Ceratitis capitata* sperm also have glycosidases on the plasma membrane with respective glycoconjugates on the egg surface (Intra et al., 2011). The presence of more than one enzyme-substrate pair could indicate that there are multiple receptors and ligands involved in the *Drosophila* sperm-egg interaction. Despite these advances, the specific details about insect sperm-egg recognition are unknown, and all studies to date have been restricted to dipterans. In order to understand the molecular basis of fertilization in insects, it is important to study multiple insect orders. Having an

understanding of multiple aspects of fertilization including sperm-egg binding in multiple insect orders would allow us to make comparisons between the different orders and potentially draw conclusions about the evolution of receptor-ligand interactions in insect fertilization.

I chose to work with *A. remigis* since the large sperm are optimal for biochemical studies allowing copious amounts of protein to be extracted and studied and sperm-egg interaction is likely to occur on the plasma membrane overlying the acrosomal region of the sperm. In most organisms, the acrosome makes up only a minor fraction of the sperm volume and this would lead to the acrosomal proteins, and the plasma membrane proteins surrounding the head, making up only a small percentage of the total sperm protein on the plasma membrane. However, in *A. remigis* the plasma membrane surrounding the acrosome makes up approximately one-half of the sperm's total surface area suggesting that if the receptor for the egg is found along the entire length of the acrosome, there will be a higher proportion of the receptor in the total whole sperm. Future studies could involve using antibodies to look at the glycosidases, which are potentially acting as receptors for the egg, distribution along the sperm. Immunofluorescence studies would allow me to determine the distribution along the sperm, whereas transmission electron microscopy would inform me of whether the glycosidases are on the plasma membrane. It is of interest to determine if the distribution of the glycosidases changes from spermatogenesis through egg entry. As discussed in the introduction, the surface area of the water strider sperm is enormous and could provide an advantage for biochemical studies of egg recognition proteins. The current information I have suggests a glycosidase

is potentially acting as a receptor. In insects, the chorion, an impenetrable shell, covers the egg. This shell protects the egg from mechanical stress and desiccation but also makes it difficult for sperm entry and, so, the egg passes through a small opening called the micropyle. Since the site of sperm entry in insects is the micropyle, I predict that a high concentration conjugate sugars would be distributed along the micropyle. Further studies will need to be done to determine if the respective glycoconjugates are present on the egg and if the glycosidases present on the sperm surface bind to the glyconjugates on the egg surface. In addition, further studies using surface plasmon resonance can be done to demonstrate that glycosidases present on the sperm surface interact with glycoconjugates present on the egg surface.

Summary

The movement of sperm through the male and female reproductive tracts until it reaches the egg was the focus of this dissertation. My results are consistent with the current knowledge of insect sperm movement from the testes to the egg. In my first data chapter, I show muscle contractions in the seminal vesicles and accessory glands of male *Culex* mosquitoes. This chapter also elucidates the neurotransmitters involved in activating muscle contractions in the seminal vesicles and accessory glands of male *Culex* mosquitoes and the pathway is involved in propagating the muscle contractions in the seminal vesicles and accessory glands of male *Culex* mosquitoes. In my second data chapter, I characterize a trypsin like molecule that is present within the male *Culex* mosquito accessory glands. This trypsin like protease is likely involved in activating

sperm motility because trypsin alone activates sperm motility and accessory gland extract activates sperm motility. In my third data chapter, I characterize glycosidase enzymes that are likely involved in the sperm-egg recognition process in *Aquarius remigis*. In my fourth data chapter, I show that three genes known to be involved in regulation of circadian rhythms are expressed in the male testes of *Culex* mosquitoes and I outline plan for further studying their role in movement from the testes to the vas deferens. Studies following sperm from production in the testes to their interaction with an egg have thus far been rare. An increased understanding of the events that take place from sperm production in the testes to their interaction with an egg can aid in the development of novel insect control methods.

References

Acott T.S. and Carr D.W. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. *Biol. Reprod.* 30: 926-35.

Baltz J. and Cardullo R. (1989). On the number and rate of formation of sperm–zona bonds in the mouse. *Gamete Research*. 24: 1-8.

Bebas P. and Cymborowski B. (1999). Effects of constant light on male sterility in Spodoptera litttoralis (Lepidoptera: Noctuidae). *Physiol. Entomol*. 24: 165–170

Bebas P., Cymborowski B. and Giebultowicz J. M. (2001). Circadian rhythm of sperm release in males of the cotton leafworm, *Spodoptera littoralis*: *in vivo* and *in vitro* study. *J. Insect Physiol*. 47: 859–866

Bebas P., Maksimiuk E., Gvakharia B. O., Cymborowski B. and Giebultowicz J. M. (2002). Circadian rhythm of glycoprotein secretion in the vas deferens of moth, *Spodoptera littoralis*. *BMC Physiology.* 2: 15

Bebas P., Cymborowski B. and Giebultowicz J. M. (2002). Circadian rhythm of acidification in insect vas deferens regulated by rhythmic expression of vacuolar H+-ATPase. *J. Exp. Biol.* 205: 37–44

Cattaneo F., Pasini M., and Perotti M. (1997). Glycosidases are present on the surface of *Drosophila melanogaster* spermatozoa. *Mol. Reprod. Dev*. 48 (2): 276-281.

Cer R., Mudunuri U., Stephens R., and Lebeda F. (2009). IC50-to-Ki: a web-based tool for converting IC50 to Ki values for inhibitors of enzyme activity and ligand binding. *Nucleic Acids Research*. 37: W441–W445.

Clark GF, Dell A. (2006). Molecular models for murine sperm-egg binding. *J. Biol. Chem.* 281:13853–13856.

Cooper T.G., Yeung C.H., Wagenfeld A., Nieschlag E., Poutanen M., Huhtaniemi and Sipila P. (2004). Mouse models of infertility due to swollen spermatozoa. *Mol. Cell Endocrinol*. 216:55–63.

Cornwall G., Tulsiani D. and Orgebin-Crist M. (1991). Inhibition of the mouse sperm surface alpha-Dmannosidase inhibits sperm-egg binding *in vitro*. *Biol. Reprod*. 44 (5): 913-921.

Cottrell G.S., Amadesi S., Schmidlin F. and Bunnett N. (2003). Protease-activated receptor 2: activation, signalling and function. *Biochemical Society Transactions*.31(6):1191–1197.

Courot M., Hocherean-de Reviers M.T. and Ortavant R. (1970). Spermatogenesis. In: A. D. Johnson, W. R. Gomes, and N. L. Van Demark (Ed.). The Testis. Vol. 1. p 339. Academic Press, New York.

Darszon A., Labarca P., Nishigaki T. and Espinosa F. (1999). Ion channels in sperm physiology. *Physiol. Rev*. 79: 481-510.

Downey J. and Lambert C. (1994). Attachment of the ascidian sperm surface egg receptor N-acetylglucosaminidase to the cell membrane. *Mol. Reprod. Dev.* 38 (4): 453-458.

Feletou M. and Vanhoutte P.M. (2000). Endothelium-dependent hyperpolarization of vascular smooth muscle cells. *Acta. Pharmacol. Sin.* 21: 1–18.

Focarelli R., La Sala G., Balasini M. and Rosati F. (2001). Carbohydrate-mediated sperm-egg interaction and species specificity: a clue from the *Unio elongatus* model. *Cells Tissues Organs*. 168: 76-81.

Friedländer M., Jeshtadi A. and Reynolds S.E. (2001). The structural mechanism of trypsin-induced intrinsic motility in *Manduca sexta* spermatozoa *in vitro*. *J. Insect Physiol.* 47: 245-55.

Giebultowicz J.M., Bell R.A. and Imberski R.B. (1988). Circadian rhythm of sperm movement in the male reproductive tract of the gypsy moth, *Lymantria dispar. J. Insect Physiol.* 34:527-532

Giebultowicz J. M. and Brooks N. L. (1998). The circadian rhythm of sperm release in the codling moth, *Cydia pomonella*. *Entomol. Exp. Appl.* 88: 229–234

Giebultowicz J. M., Ridgway R. L. and Imberski R. B. (1990). Physiological basis for sterilizing effects of constant light in *Lymantria dispar*. *Physiol. Entomol*. 15: 149–156

Glabe C. and Vacquier V. (1978). Egg surface glycoprotein receptor for sea urchin sperm bindin. *Proc. Natl. Acad. Sci. USA*. 75 (2): 881-885.

Godknecht A., and Honegger T. (1991). Isolation, Characterization, and Localization of a Sperm- Bound N-Acetylglucosaminidase that Is Indispensable for Fertilization in the Ascidian, *Phallusia mammillata. Dev. Biol*. 143: 398-407

Gvakharia B. O., Kilgore J. A., Bebas P. and Giebultowicz J. M. (2000). Temporal and spatial expression of the period gene in the reproductive system of the codling moth. *J. Biol. Rhythms*. 15: 27–35

Habermann F. and Sinowatz F. (2011). Glycobiology of Fertilization and Early Embryonic Development. In H. Gabius, The Sugar Code: Fundamentals of Glycosciences. Weinheim: Wiley-VCH Verlag GmbH and Co KGaA, Weinheim.

Hoshi M., De Santis R., Pinto M., Cotelli F. and Rosati F. (1983). Is sperm a-L-fucosidase responsible for sperm-egg binding in *Ciona intestinalis. The Sperm Cell*. 107-110.

Intra J., Caro D., Perotti M. and Pasini M. (2011). Glycosidases in the plasma membrane of *Ceratitis capitata* spermatozoa. *Insect Biochemistry and Molecular Biology*, 41: 90-100.

Intra J., Cenni F. and Perotti M. (2006). An a-L-Fucosidase Potentially Involved in Fertilization Is Present on *Drosophila* Spermatozoa Surface. *Mol. Reprod. Dev*. 73: 1149-1158.

Intra J., Cenni F., Pavesi G., Pasini M. and Perotti M. (2009). Interspecific Analysis of the Glycosidases of the Sperm Plasma Membrane in *Drosophila*. *Mol. Reprod. Dev.* 76: 85-100.

Jamieson B. (1987). The Ultrastructure and Phylogeny of Insect Spermatozoa. New York, NY: Cambridge University Press.

Jocelyn H.D. and Setchell B.P. (1972). Regnier de Graaf on the Human Reproductive Organs. *J. Reprod. Fert*. Suppl 17:1-222

Kamei N. and Glabe C. (2003). The species-specific egg receptor for sea urchin sperm adhesion is EBR1,a novel ADAMTS protein. *Genes Development*. 17 (20): 2502-2507.

Kimura T., Yasuyama K. and Yamguchi T. (1989). Proctolinergic innervation of the accessory gland in male crickets (*Gryllus bimaculatus*): detection of proctolin and some pharmacological properties of myogenically and neurogenically evoked contractions. *J. Insect Physiol*. 35: 251–264.

Kuriyama H., Kitamura K., Itoh T. and Inoue R. (1998). Physiological features of visceral smooth muscle cells, withspecial reference to receptors and ion channels. *Physiol. Rev*.
78: 811–920.

Lopez L. and Shur B. (1987). Redistribution of mouse sperm surface galactosyltransferase after the acrosome reaction. *J. Cell Biol*. 105: 1663-1670.

Lopez L., Bayna E., Litoff D., Shaper N., Shaper J. and Shur B. (1985). Receptor function of mouse sperm surface galactosyltransferase during fertilization. *J. Cell Biol.* 101: 1501-1510.

Lu Q. and Shur B.D. (1997). Sperm from β1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. *Development.* 124:4 121–4131.

Matsumoto M., Hirata J., Hirohashi N. and M H. (2002). Sperm-Egg Binding Mediated by Sperm Alpha-L-Fucosidase in The Ascidian *Halocynthia roretzi*. *Zool. Sci*. 19: 43-48.

Mengerink K. and Vacquier V. (2001). Glycobiology of Sperm-Egg Interactions in Deuterostomes. *Glycobiology.* 11 (4),: 37-43.

Minor J. E., Fromson D. R., Britten R. J. and Davidson E. H. (1991). Comparison of the bindin proteins of *Strongylocentrotus franciscanus, S. purpuratus* and *Lytechinus variegatus*: Sequences involved in the species specificity of fertilization. *Mol. Biol. Evol.* **8**: 781–795.

Minor J. E., Britten R. J. and Davidson E. H. (1993). Species specific inhibition of fertilization by a peptide derived from the sperm protein bindin. *Mol. Biol. Cell* **4**, 375–387.

Miyata H., Thaler C.D., Haimo L.T. and Cardullo R.A. (2012). Protease activation and the signal transduction pathway regulating motility in sperm from the water strider *Aquarius remigis. Cytoskeleton.* 69:207–220.

Neill A.T. and Vacquier V.D. (2004). Ligands and receptors mediating signal transduction in sea urchin spermatozoa. *Reproduction.* 127: 141-9.

Ortavant R., Courot M., and Hochereau-de Reviers M.T. (1977). Spermatogenesis in domestic animals. In: H. H. Cole and P. T. Cupps (Ed.). Reproduction in Domestic Anim

Osanai M. and Baccetti B. (1993): Two-step acquisition of motility by insect spermatozoa. *Experientia.* 49: 593-5.

Pasini, M., Cattaneo, F., Pinto, M., De Santis, R., and Perotti, M. (1999). Plasma membrane association and preliminary characterization of *Drosophila* sperm surface glycosidases. *Mol. Reprod. Dev*. 52: 166-173.

Pereira B., Abou-Haila A. and Tulsiani D. (1998). Rat Sperm Surface Mannosidase Is First Expressed on the Plasma Membrane of Testicular Germ Cells. *Biol. Reprod*. 59: 1288–1295

Perotti M. C. (2001). Male sterile mutant *casanova* gives clues to mechanisms of sperm-egg interactions in *Drosophila melanogaster*. *Mol. Reprod. Dev*. 60 (2): 248-259.

Pitts J.R., Liu C., Zhou X., Malpartida J.C. and Zwiebel L.J. (2014). Odorant receptor-mediated sperm activation in disease vector mosquitoes. *PNAS* 111(7): 2566–2571

Rankin S.M., TeBrugge V.A., Murray J.A., Schuler A.M. and Tobe S.S. (2009). Effects of selected neuropeptides, mating status and castration on male reproductive tract movements and immunolocalization of neuropeptides in earwigs. *Comparative Biochemistry and Physiology* 152: 83–90.

Riemann J.G., Thorson B.J. and Ruud R.L. (1974). Daily cycle of release of sperm from the testes Mediterranean flour moth. *J. Insect Physiol*. 20:195-207

Robaire B., Hinton B.T. and Orgrebin-Crist M.C. The Epididymis. In Neill J.D., ed. Physiology of Reproduction, Third edition. New York: Elselvier. 2006; 1071-1148.

Robaire B. and Hinton B.T. The Epididymis. From Molecules to Clinical Practice. Kluwer Academic/Plenum Publishers, New York. 2002.

Rosati F. (1985). Sperm egg interaction in ascidians. In Metz, and Monroy, Biology of fertilization. (Vol. 2, p. 361 384). New York: Academic Press.

Rosati F. and De Santis R. (1978). Studies on fertilization in the Ascidans. I. Self-sterility and specific recognition between gametes of *Ciona intestinalis. Exp. Cell Res*. 112 (1): 111-119.

Rosati F. and De Santis R. (1980). Role of the surface carbohydrates in sperm-egg interaction in *Ciona intestinalis. Nature*. 283 (5749): 762-764.

Seager S. and Slabaugh M. (2010). Organic and Biochemistry for Today (7th edition ed.).

Shur B. and Hall N. (1982). A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. *J. Cell Biol.* 95: 574-579.

Shur B. (1988). Galactosyltransferase as a recognition molecule during fertilization and development. In H. Schatten, and G. Schatten, The Molecular Biology of Fertilization. New York: Academic Press, in press.

Shur B., Rodeheffer C., Ensslin M., Lyng R. and Raymond A. (2006). Identification of novel gamete receptors that mediate sperm adhesion to the egg coat . *Mol. Cell Endo*. 250: 137-148.

Sinowatz F., Plendl J. and Kolle S. (1998). Protein-Carbohydrate Interactions During Fertilization. *Acta. Anat*. 161: 196-205.

Steers W. D. (1994). Physiology of the vas deferens. *World J. Urol*. 12: 281–285

Thaler C., Miyata H., Haimo L. and Cardullo R. (2014). Waveform Generation Is Controlled by Phosphorylation and Swimming Direction Is Controlled by Ca^{2+} in Sperm from the Mosquito *Culex quinquefasciatus. Biol. Reprod.* 89(6): 1-11

Thall A., Maly P. and Lowe J. (1995). Oocyte Gal1,3Gal Epitopes Implicated in Sperm Adhesion to the Zona Pellucida Glycoprotein ZP3 Are Not Required for Fertilization in the Mouse. *J. Biol. Chem*. 270 (37): 21437-21440.

Tian J., Gong H., Thomsen G. and Lennarz W. (1997). Gamete Interactions in *Xenopus laevis*: Identification of Sperm Binding glycoproteins in the Egg Vitelline Envelope. J. *Cell Biol*, 136, 1099-1108.

Tobback J., Boerjan B., Vandersmissen H.P. and Huybrechts R. (2011). The Circadian Clock Genes Affect Reproductive Capacity in the Desert Locust *Schistocera gregaria. Insect Biochem. Mol. Biol.* 41 (5): 313-321.

Tulsiani D., Skudlarek M., and Orgebin-Crist M. (1989). Novel C -D-Mannosidase of Rat Sperm Plasma Membranes: Characterization and Potential Role in Sperm-Egg Interactions. *J. Cell Biol.* 109: 1257-1267.

Tulsiani D., Yoshida-Komiya H., and Araki Y. (1997). Mammalian Fertilization: A Carbohydrate-Mediated Event. *Biol. Reprod*. 57: 487-494.

Turner T.T. (2008). De Graaf's Thread: The Human Epididymis. *J. Androl.* 29: 237-50

Vacquier V. and Payne J. (1973). Methods for quantitating sea urchin sperm in egg binding. *Exp. Cell Res.* 82: 227-235.

van der Horst G. and Maree L. (2009). SpermBlue†: A new universal stain for human and animal sperm, which is also amenable to automated sperm morphology analysis. *Biotechnic and Histochemistry*. 1-10.

Williams S., Xia L., Cummings R., McEver R. and Stanley P. (2007). Fertilization in mouse does not require terminal galactose or N-acetylglucosamine on the zona pellucida glycans. *J. Cell Sci.* 120: 1341-1349.

Yau M.K., Liu L. and Fairlie D.P. 2013. Toward Drugs for Protease-Activated Receptor 2 (PAR2). *J. Med. Chem*. 56: 7477−7497

Yoshida M., Kawano M. and Yoshida K. (2008). Control of sperm motility and fertility: Diverse factors and common mechanisms. *Cell Mol. Life Sci.* 65: 3446-57

Zitta K., Werthheimer E. and Miranda P. (2006). Sperm N-acetylglucosaminidase is involved in primary binding to the zona pellucida. *Mol. Hum. Reprod.* 12 (9): 557-563.

Figures and Tables.

Table 6.1. Sperm Characteristics.

	Insect	Mammal
After formation from a stem cell, gonialblast undergoes several rounds of mitosis prior to meiosis	Yes	No
Mitotic/meiotic divisions involve incomplete cytokinesis	Yes	No
Spermatogenesis occurs within encapsulated cyst	Yes	No
Aggregation/fusion of mitochondria into a nebenkern	Yes	No

Figure 6.1A. Amplification curve showing actin and *or7* are expressed in the testes and the whole female.

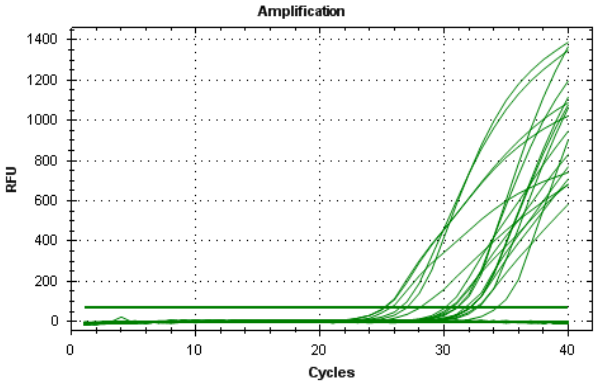


Figure 6.1B. Melt curve for *actin* and *or7*.

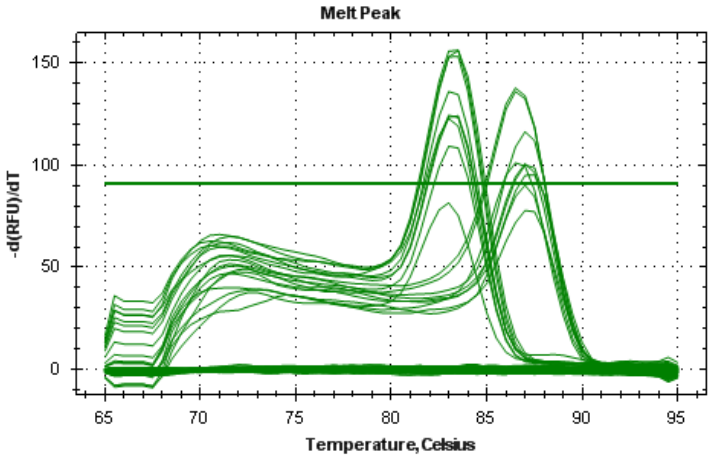


Figure 6.2. Mass spectrometry was used in an attempt to find GPCRs potentially involved in sperm motility activation. Only one GPCR was detected in the sperm proteome, odorant receptor 7 (OR7; CpipJ_CPIJ009573). OR7 is present in the sperm proteome but has a reverse topology meaning that instead of having an extracellular N-terminus, OR7 has an extracellular C-terminus. Inside the extracellular C-terminus, OR7 has potential RSLIGRL cleavage sites. OR7 is homologous to odorant receptor coreceptor (Orco) proteins in other insects. Clip art was taken Servier Medical Art Reproduction kit (http://www.servier.com/Powerpoint-image-bank) and used to produce a model of trypsin activation if OR7 is the PAR2-like protein.

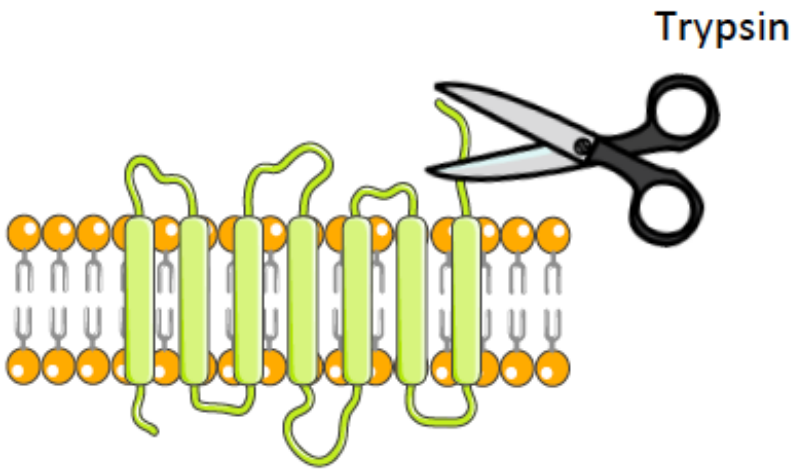
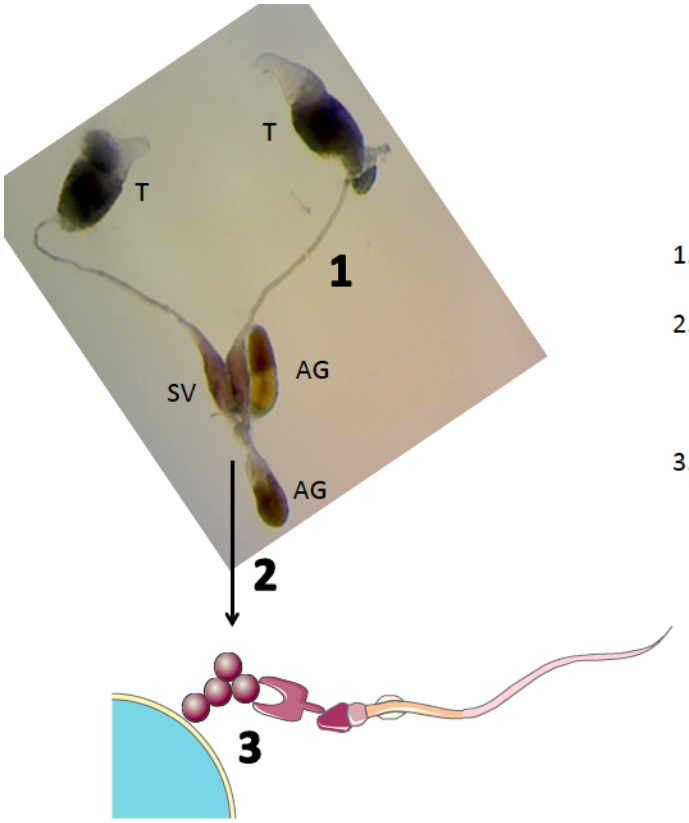


Table 6.1. Affect of inhibitors on sperm motility. 5-(N,N –hexamethylene) amiloride blocks activation of ORs and blocks the sodium-hydrogen antiporter. KB-R7943 inhibits sodium-calcium exchange.

	Motility After 10 minutes of treatment
No treatment	YES
5-(N,N – hexamethylene) amiloride	NO
KB-R7943	NO

Figure 6.3. Image of Culex male reproductive tract (taken by Dr. Catherine Thaler) used to illustrate the movement of sperm through the male reproductive tract. Diagram of sperm and egg with glycosidase and glycan, respectively, illustrating the interaction of sperm with egg.



1. Sperm move from the testis to the seminal vesicles.
2. Seminal vesicle fluid and accessory gland fluid are deposited into the female and accessory gland fluid activates sperm motility
3. Glycosidases, acting as lectins, on the sperm head recognize and bind to glycans on the egg surface.