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Calcium Homeostasis in the Activation and Regulation of Sperm Motility in the *Culex* Mosquito

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by

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ABSTRACT OF THE DISSERTATION

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by

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Insect sperm behavior and physiology are highly understudied aspect of entomology. *Culex pipiens* mosquito sperm were used to investigate the parameters of mosquito sperm activation and motility. The importance of trypsin, serine protease, calcium, and phosphorylation were examined as important components in the activation and maintenance of sperm motility. Calcium demonstrated necessity in sperm motility including but not limited to waveform generation and directional changes of the sperm. The mechanism that play a role in facilitating calcium was also investigated as without calcium, motility and waveform generation is prevented. Sperm behavior in the presence of protein specific agonists and antagonists followed a proteomic analysis of the *Culex pipiens* sperm which assisted in narrowing down proteins that play a role in calcium mobilization. In the presence of T-type voltage-gated calcium channel antagonists, sperm motility was severely inhibited. These inhibitory effects on motility suggest that a T-type voltage-gated channel is responsible for mobilizing the calcium that is necessary for sperm motility.

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Abstract

Prior to fertilization, sperm from most animals including mammals and some aquatic invertebrates are quiescent until they are activated through a variety of mechanisms. In each case, a number of post-translational modifications and calcium-dependent physiological transformations take place between the initiation of sperm motility and successful fertilization. In a variety of organisms, calcium signaling in sperm is necessary and regulated by a highly conserved and specialized voltage-gated cation channel, CatSper. Existing data has localized CatSper along the entire length of the sperm flagellum from these organisms and the absence of CatSper renders them sterile. Lower invertebrates, including the *Culex* mosquito, do not express CatSper as seen in the *Culex* proteome, but have demonstrated that calcium is necessary and sufficient and is a key component in the maintenance of sperm motility. This suggests that *Culex* sperm mobilizes calcium through another channel. Through whole sperm proteomic sequencing, it was determined there are a very limited number of proteins that may be responsible for regulating calcium. This leaves room for future research to fill our gaps in the knowledge of calcium mobilization in *Culex* sperm.

Introduction

Successful fertilization depends on a precise sequence of biochemical and physiological transformations in gametes that occur in varying environments. In animals, sperm are produced in the testis and subsequently undergo post-translational modifications resulting in the activation of mature sperm that, in part, includes a change in motility patterns. For flagellated sperm these changes in motility patterns are observed as changes in velocity, progressive motility, flagellar beat frequency and/or flagellar amplitude under the influence of extracellular biotic and/or abiotic factors. Intracellularly, changes in second messenger levels and pH are primarily initiated through an increase in intracellular Ca^{2+} through selective plasma membrane channels including, in many cases, a highly conserved and specialized calcium channel, CatSper. Many of the mechanisms leading to sperm activation are evolutionarily conserved with similar signaling pathways activated in most animal model systems including humans, mice, teleosts, and echinoderms. In contrast, arthropods (including insects) do not express CatSper, and preliminary studies have proposed an alternative mechanism for the mobilization of calcium leading to the activation of sperm motility. The aim of this paper is to review the various mechanisms that are responsible for calcium homeostasis in the control of sperm motility in a variety of animal systems.

Calcium Regulation in Mammalian Sperm

The regulation of calcium, cyclic nucleotides (cAMP and cGMP), and pH are all necessary components in the control of mammalian sperm motility. While there are other

contributing factors, these play a dominant role in the physiological transformations that occur in sperm that are necessary for fertilization including capacitation, hyperactivation, and acrosome reaction. Calcium plays many critical roles in sperm function ultimately leading to successful fertilization. Following spermiogenesis, fully formed mammalian sperm are unable to fertilize an egg and are immotile during epididymal storage (Hong et al., 1984; Setchell et al., 1993; Jones and Murdoch, 1996; Moore, 1998). After ejaculation, sperm motility is initiated yet are still not fertilization competent (Qi et al., 2007) and must first undergo a series of biochemical and physiological changes in the female reproductive tract, known collectively as capacitation.

Capacitation results from extraction of cholesterol from the sperm surface, a bicarbonate-controlled intracellular alkalinization, and a rise in intracellular Ca^{2+} (Yanagimachi, 1994; Visconti et al., 1998; Ickowicz et al., 2012). Although capacitation only requires a modest influx of intracellular calcium, it typically takes hours to complete before sperm are fertilization-competent (Fraser et al., 1998). Following capacitation, mammalian sperm flagella become hyperactivated, characterized by an increased flagellar beat amplitude and a transition from a symmetric to an asymmetric waveform. This hyperactivated motility may assist the sperm to penetrate the cumulus layer surrounding the egg and ultimately bind and penetrate the zona pellucida which is necessary before sperm-egg fusion can be achieved (Fig. 1) (Suarez et al., 2016; Yanagimachi, 1970).

Subsequent to sperm-zona pellucida binding, sperm undergo a regulated exocytotic event of the acrosomal vesicle anterior to the nucleus known as the acrosome reaction. During this exocytotic event, enzymes and other proteins within the acrosome are released resulting in modification of the zona pellucida and ultimately sperm arrival at the egg's plasma membrane (Okabe et al, 2013). The acrosome reaction occurs rapidly (within one minute), and extensive research has shown that an increase in intracellular Ca^{2+} is both necessary and sufficient for this process (Fraser et al., 1998; Michaut et al., 2000).

Numerous studies have illustrated the role that calcium plays in the regulation of motility immature mammalian sperm. Lindemann and Goltz (1988), performed a number of elegant experiments using Triton X-100-extracted epididymal rat sperm flagella that were incubated in calcium-free media containing the calcium chelator, ethylenediaminetetraacetic acid (EDTA). When the amount of free calcium fell below 100 nM, the flagellar curvature was in the same direction as the head (Lindemann and Goltz, 1988). However, as the concentration of free calcium increased from 10 μM to 100 μM, the sperm flagellum straightened before the flagellar curvature reversed direction and became tighter, forming a hook-like bend (Lindemann and Goltz, 1988). The mechanism responsible for affecting flagellar shape was investigated through the addition of sodium vanadate, a potent inhibitor of the dynein-tubulin cross bridge cycle that blocked the movement of the flagellum but did not influence the change in curvature facilitated by calcium (Lindemann and Goltz, 1988). However, the addition of cadmium, a calcium competitor/calcium channel blocker and nickel, a calcium channel blocker that

does not affect either microtubular sliding or cross-bridge cycling, diminished the motility and produced the low free calcium curvature (Lindemann and Goltz, 1988). Consequently, they concluded that the concentration of free extracellular calcium played a role in altering the motility and curvature of the flagellum in rat sperm (Lindemann and Goltz, 1988). They also confirmed that the response of the sperm to calcium was not controlled by dynein-tubulin interactions as motility occurred even in the presence of sodium vanadate (Lindemann and Goltz, 1988).

In the case of human sperm, Lee et al. (1996) also investigated the relationship of calcium and its effects on capacitation and motility. The non-specific divalent cation chelating agent, EDTA, was used to bind free calcium resulting in a true calcium-free medium and in *in vitro* conditions, the penetration of sperm past the cervical mucus and into an oocyte did not occur (Lee et al., 1996). When sperm were in the presence of EDTA for two minutes, concentrations of 5 µg/ml, 3.2 mg/ml, and 5 mg/ml, 50%, 90%, and 100% of the sperm were immotile, respectively, and total sperm motility loss occurred at a concentration of 5 mg/mL (Lee et al., 1996). However, this inhibition was reversed through the addition of calcium back into the medium (Lee et al., 1996). It was also determined that sperm-egg fusion required a calcium concentration ranging from 20-50 µM while the acrosome reaction required a higher concentration of calcium ranging from 5-10 mM (Lee et al., 1996). It was concluded that there is a linear relationship between the change in the concentration of calcium ions and the sperm motility after the exposure to EDTA (Lee et al., 1996). It also illustrates that calcium plays a necessary role

in regulating sperm motility, as well as the ability of a sperm to penetrate the cervical mucus and fertilize an oocyte (Lee et al., 1996).

The Role of Cyclic AMP in the Regulation of Mammalian Sperm Motility

In addition to calcium, the second messenger, cyclic AMP (cAMP), is involved in the regulation of mammalian sperm motility. cAMP is regulated by calcium and is produced from ATP through the action of a "soluble" adenylate cyclase (sAC) (Schlingmann et al., 2013). This increase in cAMP activates protein kinase A (PKA) (Schlingmann et al., 2013). The stimulation of PKA leads to the phosphorylation of serine/threonine and tyrosine residues resulting in hyperactivation and the induction of the acrosome reaction (Fig. 2) (Schlingmann et al., 2013).

Nolan et al. (2004) demonstrated the role of PKA in sperm function by analyzing mouse sperm that have a loss-of-function mutation in the exon of the PKA catalytic subunit $C\alpha_2$ (Nolan et al., 2004). All $C\alpha_2$ null males had normal body weight and testis weight but were infertile regardless of normal mating behavior (0/9 null males produced litters over three months) (Nolan et al., 2004). When the histology of the testes of the null males and wild-type males were compared, they were indistinguishable as sections from the epididymis contained densely packed tubules (Nolan et al., 2004). This result shows that the $C\alpha_2$ null males are still able to develop fully formed sperm (Nolan et al., 2004). The differences however were observed in the motility of the sperm as the number of motile sperm in null males was significantly decreased (Nolan et al., 2004). A significant decrease in ATP content is associated with and could contribute to this decrease in

motility (Nolan et al., 2004). When wild-type males were incubated for 60 s in a bicarbonate treatment, their motility increased 3-fold while the null male sperm displayed no change supporting the hypothesis that the activation of motility relies on PKA-mediated phosphorylation of residues on flagellar proteins (Nolan et al., 2004). These results illustrate the overall importance of the PKA subunit, $C\alpha_{2,}$ in the activation of sperm motility and phosphorylation of flagellar proteins ultimately leading to the acceleration and hyperactivation of the flagellar beat in sperm (Nolan et al., 2004).

The Regulation of Calcium through CatSper in Mammalian Sperm

CatSper is a pH sensitive, voltage gated calcium channel found exclusively in the sperm plasma membrane that is necessary for sperm motility and successful fertilization (Hwang et al., 2019). It is a complex ion channel that is encoded by at least nine different genes (Hwang et al., 2019). CatSper is mostly localized to the principal piece of the sperm flagellum and is comprised of six transmembrane proteins including four α subunits: CatSper 1-4 (of which have been reported as the most important for calcium entry and mediation of alkalinization-activated calcium selective sperm currents) and three auxiliary subunits: CatSper ß, γ, and δ (Hwang et al., 2019). In general, voltage gated, and cyclic nucleotide gated calcium channels are not directly involved in regulating sperm motility, and it is believed that only through CatSper and its regulation of cAMP-induced calcium influx can sperm motility be regulated (Ren et al., 2001). CatSper proteins are necessary for successful male fertility (Carlson et al., 2003) and

male mice that lack CatSper genes are completely sterile as their sperm cannot undergo hyperactivation (Hwang et al., 2019).

Hwang et al. (2019) explored the multi-subunit complexes in CatSper channels, specifically, the sperm specific, pH dependent calcium sensor, EF-hand calcium-binding domain-containing protein 9 (EFCAB9) that regulates CatSper's overall domain organization and channel activity. EFCAB9 is a direct binding partner of CatSper, and the protein pair acts as a "gatekeeper" prior to capacitation but stimulates the channel in response to intracellular changes in calcium and pH (Hwang et al., 2019). Using CRISPR/Cas9, EFCAB9 was knocked out in mice (Hwang et al., 2019) resulting in reduced CatSper calcium sensitivity leading to an inefficient uptake of calcium, inability for those sperm to become hyperactivated, and overall decreased motility compared to wild-type sperm (Hwang et al., 2019).

Earlier studies by Ren et al. (2001) in mice disrupted CatSper in embryonic stem cells, and the embryonic cells that carried the mutant copy of the gene were injected into blastocysts (Ren et al., 2001). These blastocysts were then implanted into pregnant mice (F_0 generation) and the mutant mice were created by mating the viable F_1 offspring. (Ren et al., 2001). The disruption of the CatSper gene caused a significant overall decrease in the activity of the sperm compared to the activity of wild-type sperm (Ren et al., 2001). The sperm from CatSper mutants were sluggish and lacked the vigorous movements characteristic of wild-type animals (Ren et al., 2001). Additionally, the sperm from CatSper mutant mice had significantly decreased fertility both *in vivo* and *in vitro* (Ren et

al., 2001), supporting a necessary role for CatSper-controlled motility for successful fertilization.

Subsequent studies showed that two isoforms of CatSper (CatSper1 and CatSper2) play separate roles in hyperactivation and progressive motility (Carlson et al., 2003; Quill et al, 2003). Mice carrying an induced Catsper1 mutation did not undergo hyperactivation (Carlson et al., 2003) while CatSper2 mutations in mice resulted in substantially decreased progressive sperm motility (Quill et al., 2003).

Qi et al. (2007) detected two more isoforms, CatSper3 and CatSper4 in the testis of mice using northern blots (Qi et al., 2007). Subsequently, CatSper3 and CatSper4 genes were deleted via homologous recombination and when these mutant female mice were mated with wild-type males, they produced a normal litter (Qi et al., 2007). However, when CatSper3 and 4 mutant males were mated with wild-type females, they did not produce a litter (Qi et al., 2007) likely because, althoughCatSper3 and CatSper4 mutant sperm were motile, they were unable to hyperactivate and undergo a downstream acrosome reaction (Qi et al., 2007) suggesting that, along with CatSper1 and CatSper2, both CatSper3 and CatSper4 are also necessary for the requisite calcium current leading to successful hyperactivated motility and fertilization (Qi et al., 2007).

Liu et al. (2007) stated that elevated extracellular levels of calcium are sufficient to induce hyperactivation in mammalian sperm (Liu, et al., 2007). However, multiple studies have shown that affecting CatSper structure or function, even in the presence of elevated calcium, is unable to induce hyperactivation. Marquez et al. (2007) showed that CatSper mutated sperm cells were not capable of hyperactivation under any physiological

conditions resulting in decreased fertility (Marquez, et al., 2007). Physiologically, CatSper is permeable to calcium and increased calcium entry leads to increased flagellar bending. If CatSper is dormant or absent, the cell will no longer possess a steady influx of calcium that would otherwise be present in a cell with a working CatSper channel (Ren et al., 2001).

The Role of pH in the Efficacy of CatSper in Mammalian Sperm

Along with second messengers, calcium and cAMP, the regulation of pH plays a significant role in Ca^{2+} homeostasis regulated by CatSper. An alkaline environment considerably increases the calcium conductance of CatSper (Qi et al., 2007). Under acidic conditions, the calcium current in wild-type mice sperm is only slightly conductive bringing only an insignificant amount of calcium into the cell (Qi et al., 2007). In mammals, as sperm move from the acidic environment of the vagina (pH 5.0) to the more alkaline environment in the cervix (pH 8.0) they go through an intracellular alkalinization leading to an increase in the conductance of CatSper resulting in enhanced calcium entry (Qi et al., 2007). This increase in intracellular calcium causes an increase in flagellar bending and hyperactivation of the sperm (Qi et al., 2007). It is thought that this hyperactive thrust of the sperm is necessary for penetration of the egg's cumulus layer and zona pellucida (Qi et al., 2007).

Kirichok et al. (2006) patch-clamped intact mouse sperm to characterize sperm calcium currents to determine the effect of pH on those currents. These investigators found that one component of the CatSper complex, CatSper1, increased in efficacy as the

intracellular pH became more alkaline (Kirichok et al., 2006). The activity of CatSper1 from the plasma membrane of mouse sperm was measured using perforated patch and whole-cell patch configurations (Kirichock et al., 2006). Using those techniques, the calcium current through CatSper1 was measured as originating from the flagellum in wild- type mice while that current was abolished in CatSper mutant mice (Kirichock et al., 2006. As the concentration of calcium in the bath solution increased from 0 mM to 10 mM at pH 8.0, there was a concomitant increase in the inward calcium current consistent with the role of an increase in intracellular calcium needed for hyperactivation and successful fertilization in the alkaline environments of the female reproductive (Kirichock et al., 2006).

Together, these results suggest that an increase in intracellular sperm pH increases calcium conductance of CatSper during capacitation (Qi et al., 2007). Similarly, Tash et al. (1989) and Ho et al. (2001) also demonstrated that CatSper became much more efficient when there was an increase in intracellular pH leading to a large influx of calcium.

Calcium and CatSper and the Importance in Teleosts

During reproduction in teleost fish, both eggs and sperm are released into the water and upon contact with the water, sperm motility is initiated (Yanagimachi et al., 2013). In most mammals and some aquatic invertebrates, sperm can penetrate the extracellular matrix surrounding the egg at any location to achieve fertilization (Yanagimachi et al., 2013). However, in teleost fish, the sperm enter the egg through a

hole in the chorion (a thick vitelline envelope) of the egg known as the micropyle, a narrow channel that gives access to the egg's plasma membrane (Yanagimachi et al., 1992; 2017). The micropyle possess thick glycoproteins known as micropylar sperm attractant (MISA) and sperm motility initiating factor (SMIF) that aid the sperm's ability to both locate and enter the micropyle on the egg in a "calcium-dependent manner" (Figure 3) (Yanagimachi et al., 2017). When flounder eggs were washed and incubated in an artificial calcium-free "seawater", the sperm swam normally as they would in normal seawater but had difficulty entering the micropyle even if they were able to contact the outer areas of the micropyle, they would swim past it (Yanagimachi et al., 2013). This led to none of the flounder eggs becoming fertilized (Yanagimachi et al., 2013). However, when the flounder eggs were placed in normal "seawater" that contained calcium, 98% of the eggs were fertilized thereby exhibiting that sperm entry into the egg via the micropyle and fertilization is calcium dependent (Yanagimachi et al., 2013). In herring sperm, calcium levels were measured using a fluorescent calcium indicator probe and fluorescence intensity before and after entering the micropylar region were compared (Yanagimachi et al., 2013). Sperm displayed a significant increase in intracellular calcium levels after entering the micropylar region of the chorion with peak pixel intensity greater than 1687 (Yanagimachi et al., 2013). This is in comparison to the sperm that only surrounded the micropyle which possessed a peak pixel intensity of less than 832 (Yanagimachi et al., 2013) The herring sperm displayed a significant increase in intracellular calcium following sperm entry into the micropyle (Yanagimachi et al., 2013). This was suggested to be due to the sperm's contact with SMIF by activating a

sodium/calcium exchanger to run in reverse thereby producing a 500% increase in the intracellular calcium concentration resulting in motility initiation (Yanagimachi et al., 2013).

As with flounder, herring sperm entry into the salmon egg's micropyle is also calcium dependent (Yanagimachi et al., 2013). When salmon eggs were isolated and inseminated in calcium-free salmon ringer the sperm entry into the micropyle was ineffective (Yanagimachi et al., 1992). The total number of sperm that moved in calcium-free salmon ringer was roughly the same as in normal salmon ringer; however, the speed was visibly slower (Yanagimachi et al., 1992). The sperm would move past the chorion in small circles and the number of sperm that entered the chorion was less than four (Yanagimachi et al., 1992). In all of these species of fish, without an influx of calcium, sperm were unable to enter the micropyle and fertilization did not occur (Yanagimachi et al., 2017). Yanagimachi et al. (2017) used immunoblots with extracts of trout and herring sperm and an antibody against CatSper3 to detect immunoreactive bands with molecular masses similar to mouse CatSper3. Along with the detection of the CatSper3 protein, immunofluorescence studies using α-CastSper3 and α-CastSper4 revealed that CatSper was localized to the midpiece in both trout and herring sperm (Yanagimachi et al., 2017). Similar to mouse sperm, CatSper in salmon sperm is necessary for sperm activation and that salmon CatSper3 has a comparable molecule mass and physico-chemical properties to mouse CatSperm3 (Lissabet, et al., 2019). Further, the CatSper inhibitor HC-056456, which binds in the pore of the center of the CatSper channel, prevents the influx of Ca^{2+} and significantly reduced the motility of salmon sperm (Lissabet et al., 2019). It is

possible that the ability of the fish sperm to reach the entrance of the micropyle is similar to sperm chemotaxis seen in echinoderms, as well as the hyperactivation of mammalian sperm during capacitation (Yanamigachi, et al., 2017).

Second Messenger Cyclic AMP in Teleost Sperm

Similar to mammalian sperm, second messengers also play an important role in the activation of motility in teleost sperm. Yanagimachi et al. (2017) investigated the effects of the cAMP-dependent protein kinase inhibitor H-89 on herring and flounder sperm to see if it would block sperm motility and sperm entry into the micropyle (Yanagimachi et al., 2017). Herring eggs and sperm were incubated in 50% artificial saltwater with or without 10 µM H-89 for ten minutes followed by attempted insemination by adding sperm to a dish containing eggs and Sperm motility and behavior around the (Yanagimachi et al., 2017). Similarly, flounder sperm were incubated in Ringer with or without 10-50 µM H-89 for ten minutes followed by incubation with eggs and observation of sperm motility and (Yanagimachi et al., 2017).

When the herring eggs and sperm were treated for five minutes in 50% ASW with 10 µM H-89, no eggs were fertilized and none of the sperm were activated around the (Yanagimachi et al., 2017). In addition, when trout sperm were incubated in potassium rich Ringer containing 10 µM H-89 for ten minutes the sperm did not initiate motility and when they were transferred to normal Ringer the sperm remained immotile (Yanagimachi et al., 2017). However, this inhibition could be reversed after several washes in normal

Ringerand sperm became fully supporting a role for p adenylyl cyclase and cAMP (Yanagimachi et al., 2017).

The Role of pH in Teleost Sperm Activation

Miura et al. (1992) investigated the effects of pH on the activation and motility of salmon sperm. In salmon, sperm taken directly from the testes are immotile (Miura et al., 1992). *In vitro* experiments were performed to determine whether pH has a direct effect on sperm motility (Miura et al., 1992). Sperm were incubated in artificial seminal plasma at a pH of 7.8 or 8.0 for 30 minutes leading to sperm motility initiation (Miura et al., 1991). The modest increase in alkalinity from pH 7.8 to pH 8.0 resulted in a significant increase in the percentage of sperm that became motile (34.9 ± 0.8% vs. 72.7 ± 14.2% respectively) with no significant change in the duration of motility (15.0 ± 2.5 sec vs. 14.7 ± 2.4 sec respectively) (Miura et al., 1992). This illustrates the importance of an alkaline environment in the ability of teleost sperm to become motile.

Calcium and CatSper Assist in Sea Urchin Sperm Chemotaxis

In the sea urchin, *Arbacia punctulata,* motile sperm are chemotactically attracted by an egg-derived peptide, resact (Seifert et al., 2015). The direction of sperm motility is regulated by calcium bursts in the flagellum that are initiated by resact binding leading to the activation of the second messenger, cGMP, and facilitated by CatSper **(**Figure 4**)** (Seifert et al., 2015).

To confirm the importance of CatSper in the mediation of calcium influxes by a chemoattractant, Seifert et al. (2015), incubated sperm in media containing caged resact and established a gradient by photolysis of the active resact in the center of an observation chamber under dark-field optics (Seifert, et al., 2015). Following resact activation, sea urchin sperm preferentially migrated to the irradiated area consistent with a chemotactic response (Seifert et al., 2015). MDL12330A and mibefradil, two CatSper inhibitors, were introduced and eliminated the resact-induced sperm congregation (Seifert et al., 2015) verifying the role of CatSper-mediated Ca^{2+} influx in sea urchin sperm chemotaxis. Further, the presence of resact also induced an increase in intracellular pH resulting in stimulating CatSper (Seifert et al., 2015). To further support the importance of alkaline pH in the regulation of calcium, Seifert et al. (2015) investigated the relationship between pH and membrane potential in CatSper activation and how a minute chemoattractant induced change in pH causes a depolarization and allows CatSper to open enabling an influx of calcium (Seifert et al., 2015). *A. punctulata* sperm, in the presence of ammonium chloride (NH_4Cl), resulted in a significant intracellular alkalinization and in increase in calcium (Seifert et al., 2015). At lower NH_4Cl concentrations (less than or equal to 3 mM), calcium levels plateaued and at higher concentrations (greater than or equal to 10 mM), there were strong oscillatory calcium responses (Seifert et al., 2015). This illustrates the role pH plays in the stimulation of CatSper and influx of calcium (Seifert et al. 2015). The CatSper gene is present in many animal genomes including humans, mice, and other vertebrates (including teleosts).

However, information regarding its presence or expression levels in other non-mammalian and invertebrate organisms, including insects, is limited.

Second Messenger cGMP in Sea Urchin Sperm

Cyclic GMP (cGMP) concentrations regulate sperm activation including motility initiation, chemotaxis, and stimulation of the acrosome reaction (Su et al., 2006). Resact, a peptide in the jelly layer of sea urchins (*Arbacia punctulata*) binds to a receptor guanylyl cyclase on the sperm flagellum (Su et al., 2006) and is a strong chemoattractant as sperm move down the resact gradient towards the egg plasma membrane (Ward et al., 1985). The rise in cGMP following guanylyl cyclase activity results in the opening of potassium channels leading to an efflux of potassium and hyperpolarization of the sperm (Su et al., 2006). This cascade of events prompts calcium oscillations in the flagellum activating motility and chemotaxis (Su et al., 2006). In order to support the importance of cGMP in sperm motility, Su et al. (2006) experimented with phosphodiesterase (PDE) inhibitors preventing the inactivation of cyclic nucleotides (cAMP and cGMP) that allows them to persist as second messengers. Sperm treated with 50 μM dipyridamole showed a substantial increase in progressive sperm motility after 10 minutes compared to controls (Su et al., 2006). Other PDE inhibitors (T-1032, zaprinase, Viagra, IBMX) also stimulated motility but their effects were less significant than with dipryridamole (Su et al., 2006).

pH and its Effect on Motility and Fertilization in Sea Urchin Sperm

In many taxa, sperm are stored in the testis or some other male accessory organ (e.g., the epididymis in mammals, the seminal vesicle in mosquitoes, etc.) (Nishigaki et al., 2014). In some of these animals, it has been argued that maintaining an intracellular acidic environment will result in the sperm being held in a dormant state because flagellar dynein typically have a pH optimum of around 8.0 (Nishigaki et al., 2014). When sea urchin sperm are dispersed into seawater (~pH 8.0), a rapid efflux of H^+ occurs increasing the intracellular pH from 7.2 to 7.6 (Nishigaki et al., 2014). This increase in intracellular then activates axonemal dynein as they approach their pH optimum. The intracellular pH and its role in motility regulation of sperm is also dependent on extracellular ionic concentrations including elevated K^+ (200mM) and Na^+-free seawater that dramatically decreases intracellular pH (5.7-6.6) resulting in a corresponding inhibition of sperm in motility (Nishigaki et al., 2014). Addition of NH_4Cl restored the alkaline environment restoring vigorous sperm motility (Nishigaki et al., 2014). Fluorescence imaging experiments have also been used to support the role of increased sperm alkalinization with motility initiation. Lee et al. (1983) used the pH sensitive probe, 9-aminoacridine, along with methylamine to measure the intracellular pH of immotile versus motile sperm. Through the use of these probes, it was established that the intracellular pH of immotile sperm (calculated from the methylamine distribution) was 6.32 ± 0.04 while the pH levels of motile sperm increased to 6.72 ± 0.11 (Lee et al., 1983). It was further determined that motility is initiated by a sodium dependent pH increase as sperm placed into artificial seawater released protons into the extracellular

environment (Lee et al., 1983). In contrast, sperm placed into sodium free artificial saltwater did not release protons thereby maintaining a lower intracellular pH holding the sperm immotile (Lee et al., 1983). Taken together, these data support a model where motility initiation is driven by an intracellular sodium-dependent alkalinization (Lee et al., 1983).

Calcium Affects Forward Motility in Insect Sperm

There are very few studies regarding the mechanisms that regulate sperm motility in insects, including mosquitoes, although calcium is a necessary component for initiation and maintenance of flagellar beating and progressive motility similar to what has been described in other animal systems (Figure 5). Thaler et al. (2013) investigated the role of calcium in sperm from the southern house mosquito, *Culex quinquefasciatus,* by introducing activating agents and Ca^{2+} chelating agents *in vitro* (Thaler et al., 2013). Sperm that were initially incubated in a calcium free, EGTA-containing isotonic solution were unable to initiate motility even in the presence of trypsin (Thaler et al., 2013), a serine protease found in the accessory glands that is the sperm motility-activating agent (Thaler et al., 2015; Stephens et al., 2018). This raises the possibility that an activator like a serine protease requires calcium to initiate motility (Thaler et al., 2015). In order to eliminate this possibility, mosquito sperm that were incubated with trypsin in a calcium-free isotonic solution and could not initiate motility (Thaler et al., 2015). Only when the sperm were incubated in a solution containing both calcium and trypsin was motility initiated (Thaler et al., 2015). Interestingly, proteomic sequencing of Culex sperm reveals

that CatSper is not present in the proteome of *Culex* (Thaler et al., submitted). It is possible that the CatSper complex vanished or was never conserved. In aquatic animals with external fertilization, including some teleost fish and echinoderms, CatSper plays a key role in chemotaxis and the ability of the sperm to locate the oocyte (Lishko et al., 2010). In birds, the oocyte has a very thin wall and the hyperactivation of the sperm to penetrate the egg might be unnecessary, negating the need for a CatSper-controlled calcium rise in the flagellum (Lishko et al., 2010). It is also possible that calcium increases may be derived from other sources including other calcium-conducting mechanisms through the plasma membrane or via intracellular stores that have taken the place of the CatSper complex (Cai et al., 2008). At this point, it is unclear whether insect sperm rely on pH changes or other second messengers to manage their motility as they would if they possess CatSper.

Odorant Coreceptor in *Anopheles gambiae* Sperm

It is unknown how insect sperm regulate their calcium. While there may be conserved proteins across insect species that play a role in mobilizing calcium, each species may have its own mechanism, and non-mammalian organisms in general may use different mechanisms. One receptor under consideration is Orco, typically associated with olfaction in insects. Insects possess olfactory receptors that are heteromeric, ligand gated, seven transmembrane ion channels that normally comprise an odorant specific binding subunit (OR) and a coreceptor subunit (Orco) (Guo et al., 2018). Together they form the complex Or/Orco, a cation channel that allows an influx of calcium (Guo et al.,

2018). However, Jones et al. (2011) discovered that the Orco subunit can form a functional cation channel without the presence of the OR subunit and can be gated by the agonist VUAA1 (Jones et al., 2018). Orco is made up of seven-membrane traversing helical segments (S1-S7) with an intracellular amino terminus and an extracellular carboxy terminus (Butterwick et al., 2018). As a complex, it forms a tetrameric protein with four subunits surrounding a pore (Butterwick et al., 2018). Pitts et al. (2014) discovered the existence of Orco in the sperm flagella of one mosquito species, *Anopheles gambiae*, and RNA sequencing was used to quantify transcript numbers in *A. gambiae* testis (Pitts et al., 2014). An Orco antibody was utilized to investigate the protein expression of *Anopheles gambiae* Orco (AgOrco) in the testes and found that the AgOrco protein is localized along the flagella of fully developed sperm (Pitts et al. 2014). In support of AgOrco's role in sperm motility, flagellar beating significantly increased when AgOrco was activated in the presence of two Orco agonists, VUAA1 (the agonist that could be a potential gate for Orco) and VUAA4 (Pitts, et al., 2014). However, in the presence of the AgOrco antagonist, VU0183254, flagellar beating did not occur (Pitts et al., 2014). When the agonists were introduced along with the antagonist, the activation of flagellar beating was substantially lowered (Pitts et al., 2014). To further investigate the roles of agonists on flagellar activation and beating, Pitts et al. (2014) used an Orco mutant strain of *Aedes aegypti* (Pitts et al., 2014). When the wild-type strain was presented with an agonist, there was a successful activation and maintenance of sperm motility while Orco mutants were unresponsive (Pitts et al., 2014). This study suggests that because mosquito sperm do not express CatSper it is likely that another protein(s)

and mechanism is responsible for facilitating the calcium that plays a role in sperm motility and forward progressive movement. In *Culex pipiens* sperm, whether that is Orco or another protein(s) remains to be investigated.

Discussion

There is a great deal of published data in support of calcium's role in a number of different physiological events in sperm that lead to successful fertilization in mammals, teleost fish, sea urchins, and insects. In mammals it is known that sperm are immotile in the epididymis prior to ejaculation and although motile, are still unable to successfully fertilize an oocyte. A controlled influx of calcium is needed to initiate capacitation, hyperactivation, and the acrosome reaction which are all necessary prior to sperm-egg fusion. Lindemann and Goltz (1988) concluded that the concentration of free extracellular calcium plays a role in altering the motility and curvature of the flagellum in rat sperm (Lindemann and Goltz, 1988). They also confirmed that the response of the sperm to calcium was not controlled by dynein-tubulin interactions as motility occurred even in the presence of sodium vanadate (Lindemann and Goltz, 1988). In teleost fish, Yanagimachi et al. (2017) illustrated sperm's ability to hyperactivate to locate and use chemotaxis to enter a micropyle is calcium dependent based on their ability to do so when incubated in calcium containing solution and absence in calcium-free solution. In sea urchin calcium bursts as a result of high cyclic GMP is what leads to motility and eventually fertilization. Insects also use calcium to initiate motility and when in the presence of chelator, EDTA, the sperm were unable to initiate flagellar beating.

In many animals, CatSper is a pH sensitive, and voltage gated plasma membrane channel located on the principal piece of sperm where it is the main mediator of calcium flux. Numerous studies support a role for CatSper in successful fertilization since mice with compromised or inactive CatSper are infertile. This is likely because decreased calcium influx mediated by CatSper prevents the downstream activation of second messengers that stimulate capacitation, hypermotility, chemotaxis, and the acrosome reaction. Second messengers have also been shown to play a role in the control of flagellar beating in a number of animals including mammals, teleost fish, and sea urchins. In mammals, an increase in intracellular calcium and levels of cAMP increases flagellar amplitude and regulates motility through the action of other downstream proteins such as protein kinase A (PKA) (Schlingmann et al., 2013). Experiments have proven that mice that lack the subunit $C\alpha_2$ are infertile demonstrating its necessity to fertilization. As in mammals, teleost fish also possess cAMP that is regulated by an influx of calcium. When calcium is available, it causes cAMP to activate PKA which leads hyperactivated sperm toward the micropyle that then enter, thereby fertilizing the egg. To further support this, when a cAMP-dependent protein kinase inhibitor, none of the fish eggs were fertilized (Yanagimachi et al., 2017). Sea urchin on the other hand use the effect of calcium on cGMP to activate motility, chemotaxis, and fertilization. The sea urchin specific chemoattractant resact diffuses from eggs of female sea urchins (Ward et al., 1985). Resact then binds to its receptor causing an increase of cGMP which downstream prompts calcium oscillations in the flagellum causing hyperactivation towards the egg (Ward et al., 1985; Su et al., 2006). When the sperm were treated with a

phosphodiesterase inhibitor, the cGMP levels were maintained and caused a six-fold increase in motility (Su et al., 2006).

Besides the similar need for calcium among mammals, teleost fish, and sea urchin, an alkalinized environment is crucial for the functioning of CatSper. It is likely that the reason sperm remain dormant prior to release is because of the extremely acidic environment which is below the pH optimum of sperm dynein ATPases (Nishigaki et al., 2014; Qi et al., 2007). In mammals, as sperm move up the female reproductive tract, they go from an area of high acidity to a more alkalinized area, resulting in the activation of CatSper and, ultimately, hyperactivation. In teleost fish, Oda et al. (2017) through a series of experiments confirmed that a high intracellular pH was indeed important in the activation and initiation of motility in the sperm and that sea urchin's motility is inhibited when the intracellular pH is lowered.

To date, there is a lack of published research regarding the effect of second messengers and pH on insect sperm physiology including how calcium influx is regulated. It is plausible that their absence of CatSper led to the evolutionary emergence of another protein capable of facilitating the calcium needed in sperm motility. These data suggest that instead of CatSper, other proteins may play a role in mobilizing the calcium that insects sperm require for successful fertilization. Whether these protein, or an as of yet identified calcium conducting mechanism, plays a role in insect sperm calcium homeostasis, remains to be seen.

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Figures and Tables

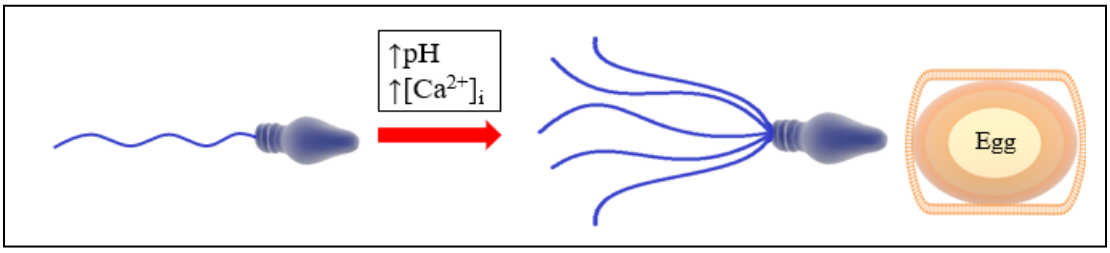

Figure 1.1. Key signaling events in mouse sperm. Initially sperm are quiescent in the epididymis and after ejaculation. Movement from the vagina into the cervix creates an increase in pH stimulating the opening of CatSper and an influx of calcium. Following the influx of calcium, the sperm become capacitated and hyperactive allowing them to successfully fuse to and penetrate the plasma membrane in an acrosome reaction, fertilizing the oocyte.

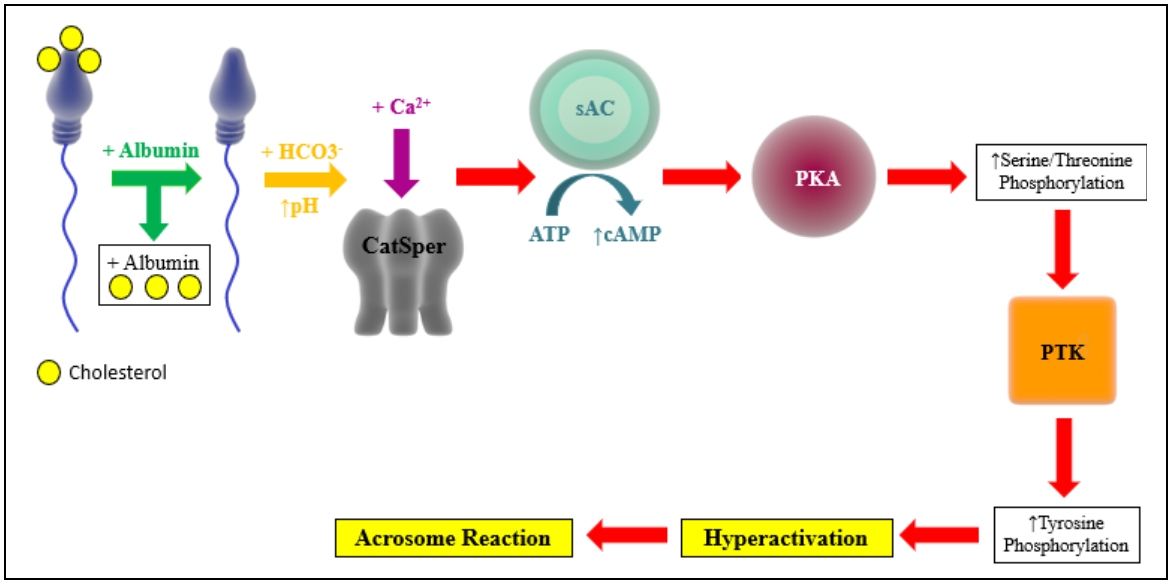

Figure 1.2. Key physiological transformations and signaling pathways in mammalian sperm leading to successful fertilization. Mammalian sperm contain cholesterol on the sperm surface. When the sperm come into contact with albumin in the female reproductive tract, the cholesterol is removed increasing membrane fluidity and allowing bicarbonate to enter causing a bicarbonate-controlled intracellular alkalinization and an increase in pH. This stimulates the opening of CatSper which causes an influx of calcium and the stimulation of soluble adenylyl cyclase to catalyze the conversion of ATP into cAMP. This cAMP increase leads to post-translational modifications by protein kinase A (PKA) and protein tyrosine kinase (PTK) leading to downstream physiological transformations in the sperm including hyperactivated sperm motility and the acrosome reaction.

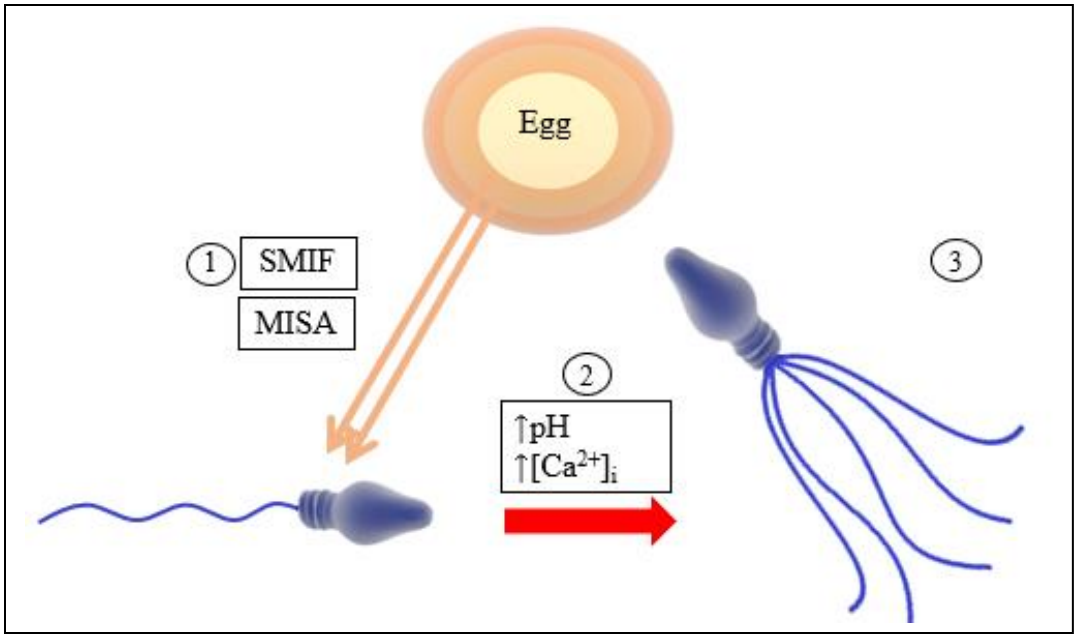

Figure 1.3. Key signaling events in teleost sperm. In the presence of the chemoattractants sperm motility initiating factor (SMIF) and the micropylar sperm attractant (MISA), there is an increase in intracellular pH that stimulates the opening of CatSper The resulting influx of calcium, stimulates motility and the chemoattractant orients the sperm towards the egg which resulting in successful fertilization of the egg.

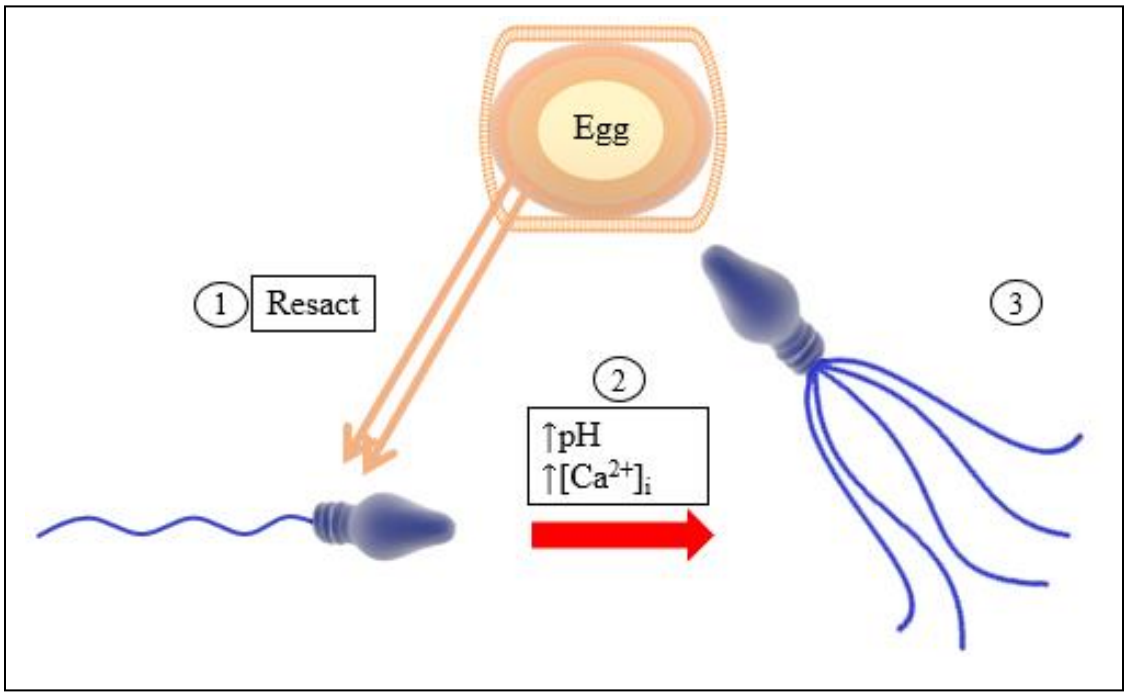

Figure 1.4. Key signaling events in sea urchin sperm. In the presence of the egg-derived egg peptide and chemoattractant, resact, there is an increase in intracellular pH which stimulates the opening of CatSper in the sperm. This results in an influx of calcium leading to flagellar hyperactivity and orientation towards the egg ending with an acrosome reaction and successful fertilization of the egg.

Calcium Channels				
Organism	**Type of Channel**	**Location on Sperm**	**Function**	**Effect when Absent**
Mice	CatSper	Principal Piece	Capacitation, hyperactivation, acrosome reaction, and fertilization.	Sterility
Humans	CatSper	Principal Piece	Capacitation, hyperactivation, acrosome reaction, and fertilization.	Sterility
Herring	CatSper	Midpiece	Sperm motility, locating and penetrating micropyle, and fertilization.	Sterility
Flounder	CatSper	Midpiece	Sperm motility, locating and penetrating micropyle, and fertilization.	Sterility
Salmon	CatSper	Midpiece	Sperm motility, locating and penetrating micropyle, and fertilization.	Sterility
Trout	CatSper	Midpiece	Sperm motility, locating and penetrating micropyle, and fertilization.	Sterility
Sea Urchin	CatSper	Principal Piece	Chemotaxis and ability to locate oocyte.	Sterility
Insect	Unknown	Unknown	Unknown	Unknown

Table 1.1. Summary of various organisms' mechanisms to mobilize calcium in sperm.

Chapter 2: The Role that Trypsin and Calcium Play in the Activation and Maintenance of Sperm Motility

Abstract

In a variety of animal systems, it has been demonstrated that calcium is both necessary and sufficient for the regulation of sperm motility and, ultimately, for successful fertilization. Lower invertebrates, including the *Culex* mosquito, also require calcium to maintain their sperm motility. Alongside the importance of calcium in the maintenance of sperm motility, there also exists an endogenous activator and downstream phosphorylation events that play a role in both sperm activation of flagellar waveform development. Although calcium is necessary for sperm motility in many organisms, including *Culex* mosquitoes, the mechanisms for calcium mobilization differ. In many animals, the activation of sperm motility is accompanied by changes in pH and membrane potential, as well as other molecular and cellular transformations that occur during migration through the female reproductive tract. Immotile *Culex* sperm from the seminal vesicle are activated through the action of a serine protease, trypsin, that is stored in the male's accessory glands. During ejaculation, the trypsin catalyzes the hydrolysis of a protease activated receptor that both mobilizes calcium and initiates a downstream phosphorylation cascade that ultimately initiates sperm motility. The significance of trypsin in the activation of sperm motility and calcium in the maintenance of sperm motility was demonstrated through a series of *in vitro* assays. Fully mature and immotile sperm stored in the seminal vesicles were incubated in various solutions to test the importance of trypsin and calcium on the activation and regulation of sperm motility. Both are needed to ensure successful fertilization.

Introduction

In contrast to mammals and some marine invertebrate models, little is known about sperm motility in insects, including mosquitoes, except for the calcium requirement. Many sperm are quiescent prior to fertilization (Vines et al., 2002; Ohmuro et al., 2004; Miyata et al., 2012) presumably to conserve energy during sperm storage. Sperm require an activation mechanism to transition from quiescent to motile (Miyata et al., 2012). In the silk moth, *Bombyx mori*, sperm were activated when they were mixed with seminal fluid containing prostatic gland extracts suggesting that these secretions initiate a signaling cascade to activate the sperm (Nagaoka et al., 2012). *Bombyx mori* produces two types of sperm, anucleated (apyrene) and nucleated (eupyrene) both of which are immotile in the seminal vesicle (Nagaoka et al., 2012). In the presence of the prostatic gland secretions, apyrene sperm were stimulated and eupyrene sperm bundles were dissociated (Osanai and Baccetti, 1993; Nagaoka et al., 2012). These secretions were named initiatorin and determined to be a type of serine protease since the effects could be mimicked by other proteases such as trypsin and endoproteinase Arg-C (Nagaoka et al., 2012). Orthopteran species only possess one type of sperm, eupyrene (Osanai and Baccetti, 1993). Sperm motility in Orthoptera was also initiated by a serine protease, trypsin, similar to sperm activation in *B. mori* and is enhanced by cAMP (Osanai and Bacetti, 1993). Unfortunately, there have been very few studies regarding sperm activation and signaling in insect sperm. However, the results regarding Lepidopteran and Orthopteran insects suggests that there is a common and conserved

signaling pathway involving proteases that leads to the activation of insect sperm (Miyata et al., 2012; Thaler et al., 2013).

The water strider, *Aquarius regimis* also experienced fully activated sperm in the presence of the serine protease, trypsin (Miyata et al., 2012). When *A. regimis* sperm were activated in the presence of trypsin, the sperm flagellum displayed a sinusoidal waveform (Miyata et al., 2012). When the sperm were incubated in papain, they became motile but at a much slower rate than trypsin (Miyata et al., 2012). Thrombin, a serine protease that activates platelets, was also investigated, but did not activate sperm motility at even high concentrations suggesting that proteolysis at a specific site is required for mosquito sperm motility activation (Miyata et al., 2012). In addition to the importance of protease activity, downstream phosphorylation events also play a role in sperm motility.

Based on previous data, most flagellar motility in sperm is also regulated by protein phosphorylation (Visconti and Kopf, 1998; Urner and Sakkas, 2003; Neill and Vacquier, 2004; Miyata et al., 2012;). To determine if trypsin activation is mediated by protein kinases, mature *A. regimis* sperm were incubated with a broad-spectrum kinase inhibitor, staurosporine (Miyata et al., 2012). In the presence of both trypsin and staurosporine, the general level of motility decreased in a concentration dependent manner (Miyata et al., 2012). When the sperm were incubated with 20 µM staurosporine, they were completely immotile while control sperm remained motile for 45 minutes (Miyata et al., 2012). These data suggest that kinase activity is necessary for the maintenance of sperm motility in *A. regimis* (Miyata et al., 2012).

Treatments that increase intracellular calcium can stimulate the activation of some kinases including CamKII and PKC (Miyata et al., 2012). Mature *A regimis* sperm were incubated in ionophore A23187 and calcium, or in the SERCA inhibitor thapsigargin, in order to investigate the role of increasing intracellular calcium on sperm motility (Miyata et al., 2012). Treatment with A23187 and calcium partially activated sperm motility while treatment with thapsigargin considerably activated sperm motility (Miyata et al., 2012). However, both treatments failed to stimulate sustained motility suggesting that only raising intracellular calcium is not enough to stimulate fully the signaling pathway (Miyata et al., 2012).

To determine whether calcium is necessary for the maintenance of sperm motility in *A. regimis*, mature sperm were pre-incubated in BAPTA-AM, an intracellular calcium chelator, and then were exposed to trypsin (Miyata et al., 2012). Sperm in the presence of low concentrations of BAPTA-AM achieved substantial motility although not quite at the same level of the control sperm (Miyata et al., 2012). When the BAPTA-AM concentration was increased, motility levels significantly decreased (Miyata et al., 2012). Sperm were then re-incubated in 20 µM BAPTA-AM and were introduced to a solution containing trypsin 10 µM A23187, and 100µM calcium (Miyata et al., 2012). When the calcium was added to the sperm treated with BAPTA-AM, it significantly increased the percent of motile sperm suggesting that calcium is required for sperm motility (Miyata et al., 2012).

The roles of PKC and CamKII were investigated to see if they act downstream of calcium. *A regimis s*perm were treated with the PKC activator SC-10 that only generated

minimal motility (Miyata et al., 2012). The PKC inhibitor Go6983 did not prevent trypsin-stimulated motility, while the PKC inhibitor Calphostin C increased the lag time for the stimulation of sperm motility but overall did not decrease motility (Miyata et al., 2012). The CamKII inhibitor KN-93 also increased the lag but again did not reduce sperm motility (Miyata et al., 2012). These results imply that PKC and CamKII may affect the activation of sperm motility (Miyata et al., 2012). Following these findings, cyclic nucleotide dependent protein kinases and their role on sperm activation were investigated (Miyata et al., 2012). High concentrations of 8-bromo-cAMP but not 8-bromo-cGMP considerably increased sperm motility although not to the same level of trypsin activated sperm motility (Miyata et al., 2012). Treatment with the PKG and PKA inhibitor, H8, did not reduce trypsin activated motility (Miyata et al., 2012). However, when the MEK1/2 inhibitor U0126 was tested, it substantially increased activation lag time of the sperm and drastically decreased the percentage of motile sperm (Miyata et al., 2012). These results suggest that cAMP can moderately stimulate motility, PKG and PKA are not involved, and that activation of the MAP kinase (MAPK) pathway is significant in the stimulation of sperm motility (Miyata et al., 2012).

Collectively, these results in *A. regimis* demonstrate that sperm motility is activated by trypsin and maintained by an increase in intracellular calcium and MAPK signaling (Miyata et al., 2012). Activation is likely due to the stimulation of a class of protease activated receptors (PARs) which are part of a small family of G-protein coupled receptors seen in other organisms (Miyata et al., 2012). These include PARs 1-4, of which only PAR2 is activated by trypsin while PAR1,3 and 4 are activated by

thrombin (Miyata et al., 2012). Trypsin catalyzes the cleavage of the amino terminus on a PAR2 protein stimulating a signaling cascade which leads to the activation of a MAPK pathway and ultimately the stimulation of sperm motility through the phosphorylation of axonemal proteins (Neill and Vacquier, 2004; Miyata et al., 2012).

Following the discovery that serine proteases activate sperm motility in insects and the significance of phosphorylation in flagellar motility, Thaler et al. (2013) investigated the role of accessory gland components and sperm motility in mature *Culex* sperm to determine if motility is initiated and maintained similarly in Hemi- and Holometabolous insects. When mature sperm from the seminal vesicle were incubated in the presence of accessory glands components, motility was initiated as a progression of waveform transitions over time (Thaler et al., 2013). These flagellar waveforms consisted of waveform A, a low-amplitude, long-wavelength waveform, waveform B, a low-amplitude, short-wavelength superimposed on a high-amplitude, long-wavelength waveform, and waveform C, a double waveform converted into a single helical wave (Thaler et al., 2013). As these wavelengths progressed from A to C, both the sperm velocity and the progressive motility increased (Thaler et al., 2013). When mature sperm were incubated only in an isotonic medium (Insect Ringer) the progression of waveforms did not occur with most sperm remaining immotile or displaying only a nonprogressive and weak waveform A for ten minutes (Thaler et al., 2013). These experiments suggested that the accessory glands contain the components that activate the sperm (Thaler et al., 2013).

As previously seen in Lepidopterans and Orthopterans, the presence of serine proteases is sufficient to initiate sperm motility in calcium containing media (Thaler et al., 2013). To support this, *Culex* sperm that were incubated in accessory gland fluids and a serine protease inhibitor, aprotinin, displayed only weak activation with less than 10% of the sperm reaching waveform B and 1% of the sperm reaching waveform A (Thaler et al., 2013). When sperm were incubated in the absence of accessory glands but in the presence of 2 µg/ml trypsin, the sperm were fully activated and the results were similar to the activation via accessory gland components (Thaler et al., 2013). This is a result of the accessory glands containing numerous proteases including four isoforms of trypsin as well as nine serine protease inhibitors determined through mass spectrometry (Stephens et al., 2018). These serine proteases are potential activators of sperm motility as accessory gland extracts or trypsin are sufficient to activate sperm motility while the serine protease inhibitors are present in order to inactivate the stores proteases (Stephens et al., 2018).

Following trypsin activation, downstream signaling and kinase activity was investigated. When mature *Culex* sperm in the presence of accessory gland components were incubated with either a broad-spectrum protein kinase inhibitor, staurosporine, or a tyrosine kinase inhibitor, genistein, the development of waveform A and the transition to waveform B was unaffected (Thaler et al., 2013) although they did inhibit the transition from waveform B to waveform C (Thaler et al., 2013). Additional pharmacological studies support a model where the accessory gland components activate a MAPK pathway and that phosphorylation via MAPK is required for the transition from

waveform B to waveform C (Thaler et al., 2013). Therefore, similar to both Hemi- and Holometabolous insects, serine protease directed protein phosphorylation in mosquitoes plays a significant role in the activation and maintenance of sperm flagellar motility.

In this chapter, I expand on these experiments to determine the role that trypsin plays in the activation of sperm motility, the importance of calcium and the effect on sperm motility when reduced or absent, and how phosphorylation effects sperm activation and flagellar behavior.

Materials and Methods

Chemicals and Reagents

EGTA, okadaic acid, and trypsin were purchased from Sigma Aldrich (Burlington, MA).

Insect Sperm Media

Insect Ringer contained 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 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. Calcium Free Insect Ringer (CFR) contained 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, 4 mM EGTA, pH 7.2. Phosphate Free Insect Ringer (PFR) contained 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 1 mM glucose, 20 mM HEPES, pH 7.2. Insect Ringer that was free of phosphates and sulfates (PSFR) contained 110 mM NaCl, 7 mM KCL, 2.4 mM $MgCl_2$, 1.2 mM $NaHCO_3$, 1 mM glucose, 20 mM HEPES, 4 mM EGTA, pH 7.2.

Mosquitoes and Dissections

The *Culex pipiens* wild-type line was maintained at 27°C, 80% relative humidity, 12:12 light/dark cycle, and given sugar cubes as a nutrient source. The *C. pipiens* line is autogenous, meaning a blood meal is not required for the females to lay egg rafts, therefore only sugar was provided for the females. Egg rafts were collected in trays of

recycled larval water and were hatched in cups containing deionized water where they were then placed into larger pans. The larvae were fed on a 2:1 diet of mouse food and brewer's yeast with the addition of 1.8 g fish oil per 25 g mouse food and brewer's yeast. Pupae were collected using a sieve and added into cups with the remainder of the larvae water. Cups were added into mosquito cages and allowed to eclose for four days. On the fourth day, the pupae cups were strained into oviposition trays which remained in the mosquito cages for two days to allow females to lay eggs. The adult mosquitoes were collected using a battery powered aspirator and were anesthetized and sorted under CO_2. Males were then placed individually in 1.25-ounce Solo cups (product #P125, Solo Cup Company) with lids (product #PL1, Solo Cup Company, Lake Forest, IL). Chloroform-soaked cotton balls were introduced to the cups to euthanize the mosquitoes. The male reproductive tract was then dissected out in either phosphate buffered saline (PBS): 20 mM Na_2HPO_4, 100 mM Na_2HPO_4 or with the appropriate Insect Ringer and placed on a clean glass side with the identical Insect Ringer. All sperm used in these experiments were mature sperm located in the seminal vesicles. Unless otherwise stated, testes and accessory glands were removed prior to the assay leaving only the seminal vesicles to prevent the initiation of motility until an appropriate activator was added to the slide.

Sperm Motility Assay

To quantify sperm motility under various conditions, sperm from the seminal vesicles were placed in a total of 20 µl of the appropriate Insect Ringer (e.g., Insect Ringer, calcium-free Insect Ringer containing EGTA (CFR), Insect Ringer without

phosphates (PFR), and Insect Ringer without phosphates, sulfates, and adjusted KCl and $MgCl_2$ (PSFR)). The seminal vesicles were squeezed open using forceps into the appropriate media to release the sperm. A coverslip with clay at each corner was added to the top of the slide and pressed down until the coverslip touched the liquid. This allowed the sperm to move freely in three dimensions after motility was initiated with the appropriate activator. The slide was then moved to a microscope stage and sperm motility was monitored on a darkfield microscope (Nikon Labophot, Melville, NY) and images were captured using a high-speed camera (Edgertronic, SC1, Campbell, CA) using a 20X objective lens at 100 frames/sec for 10 seconds resulting in a compilation of 1,000 images.

In control assays, sperm were incubated for 5 minutes in either Insect Ringer or CFR both of which contained the endogenous activator, trypsin at a working concentration of 2 µg/ml (Table 2.1). Fields containing numerous sperm were recorded for 10 seconds at 100 frames/second. During the 10 second recordings, the microscope stage was moved to three different fields of view to provide data for different sperm behaviors in the solution. Total sperm were calculated for each field of view and motile and immotile sperm were also counted and averaged over the three different views. Motile sperm were scored as sperm displaying either flagellar beating or forward progression while immotile sperm were sperm that displayed no flagellar motion. Total sperm counts were performed using image processing software (ImageJ, NIH). Counts were repeated for each biological replicate. The total number of sperm counted in each condition change ranged from ~150-400.

Statistical Analysis

All data were analyzed using the statistical software on SigmaPlot 14.5 (Systat Software Inc., San Jose, CA). Welch's t-test was used to compare the data between two groups, the control and one treatment at a time. Equal variances were not assumed. P-values: **** < 0.0001, *** 0.0001 to 0.001, ** 0.001 to 0.01, * 0.01 to 0.05, were considered significant and ns ≥ 0.05.

Results

Mosquito Sperm Motility Activation Requires Trypsin in the Presence of Calcium

To investigate the importance of trypsin and calcium in sperm motility, a series of *in vitro* assays were performed. Seminal vesicle dissections were performed to release mature sperm from the *Culex* male mosquito, which were incubated in various solutions. To investigate these varying conditions, both trypsin and calcium were manipulated in the solution. Sperm incubated in the presence of Insect Ringer containing calcium and trypsin, an endogenous activator of sperm motility, displayed nearly 100% flagellar beating, as well as successful waveform transitions leading to robust forward progressive motility (Table 2.2). When only trypsin was removed from the Insect Ringer, which still contained calcium, most sperm were immotile with only 14% achieving motility with little forward progressive movement. These data indicate that in the presence of calcium, trypsin is necessary for the initiation of *Culex* sperm motility.

Calcium is Necessary for the Activation of Mosquito Sperm Motility in the Presence of Trypsin

In order to understand the role calcium plays in sperm motility, sperm were incubated in a calcium free, EGTA-containing Ringer (CFR) without trypsin but in the presence of accessory gland components which contains the endogenous serine proteases. Under these conditions, in the presence of the accessory glad components, only 1% of the sperm displayed forward progression and motility. Further, when the accessory gland components were replaced with trypsin as an alternative activator, sperm motility

remained strongly inhibited. When trypsin was added back into the CFR, sperm motility marginally increased to 18%. It was not until both trypsin and 5 mM calcium were added back into the Insect Ringer solution, was sperm motility was fully restored (95%) (Figure 2.1). These data suggest that both trypsin and calcium are needed to ensure motility in sperm.

Increased Overall Phosphorylation is Sufficient to Activate Mosquito Sperm Motility

Along with trypsin and calcium, phosphorylation as mentioned previously, also plays a role in *Culex* sperm motility and to demonstrate the importance of phosphorylation, okadaic acid, a phosphatase inhibitor, was utilized. When sperm were incubated in the presence of okadaic acid and Insect Ringer containing calcium, but without an activator, the sperm were motile (88%) suggests that an increase in phosphorylation can bypass trypsin activation (Figure 2.2). When sperm were incubated in the presence of CFR with EGTA and okadaic acid, most sperm were motile (83%) however, and all motile sperm displayed backwards motility.

Discussion

During storage in the seminal vesicles, mosquito sperm are quiescent and only following ejaculation and the mixing of components in the accessory glands is sperm motility initiated. When *B. mori* sperm were incubated in the presence of prostatic gland extracts, they became activated suggesting that these secretions contained a signaling cascade initiator, originally named initiatorin (Nagaoka et al., 2012). Additional studies revealed that these effects could also be achieved through the action of serine proteases, including trypsin (Miyata et al., 2012). Similar studies in Orthopterans and Lepidopterans also revealed that the presence of serine proteases are sufficient to activate sperm motility suggesting that there is an evolutionarily conserved signaling pathway for the activation of insect sperm motility (Miyata et al., 2012; Thaler et al., 2013). Sperm from the water strider, *A. regimis*, also experienced fully activated sperm in the presence of the serine protease, trypsin (Miyata et al., 2012). In *A. remigis* sperm, the activation of sperm by trypsin is consistent with the presence of a PAR2, a member of a family of G-protein coupled receptors that leads to the activation of signaling pathways in specific cell types in vertebrates. In water striders, the current model suggests that the activation of a sperm specific PAR2, results in the autohydrolysis of its amino terminus on the protein, and in the presence of calcium, stimulates a calcium dependent protein kinase (PKC), that stimulates a phosphorylation cascaded ultimately leading to the phosphorylation of a yet identified target within the flagellar axoneme resulting in changes in the flagellar waveform.

Culex sperm are also activated by a serine protease and although not confirmed, likely utilize the PAR2 signaling pathway in sperm motility activation. Indeed, Stephens et al. (2017) examined the components of *Culex* accessory glands and identified the presence of 13 serine proteases and nine serine protease inhibitors using mass spectrometry that play a role in the activation of sperm motility that was investigated in this chapter.

Earlier findings in *Culex* were expanded by investigating the addition of calcium following activation through trypsin. When mosquito sperm were incubated in both trypsin and calcium, there was a rapid increase in forward progressive motility. When the trypsin was removed, both overall sperm motility and flagellar waveform development were dramatically reduced. In the absence of both trypsin and extracellular calcium, sperm motility was completely abolished but these were both fully restored when they were reintroduced to the sperm. Taken together, these data support a model whereby the presence of both a serine protease and calcium are necessary to initiate and maintain motility in mosquito sperm.

Protein phosphorylation, as seen in other organisms was also determined to play a role in *Culex* sperm motility. By increasing the overall phosphorylation state through the action of only a phosphatase inhibitor in the presence of calcium, most sperm became vigorously motile suggesting that phosphorylation of key targets in the axoneme are sufficient to initiate and maintain flagellar waveforms leading to progressive motility and that these phosphorylation events occur downstream of the trypsin-mediated activation. As seen previously, when calcium was removed from the media, the increased

phosphorylation state resulted in an interesting behavior, the sperm swam backwards Thaler et al., 2013). To date, this behavior has only been seen in one other species, *Drosophila melanogaster. D. melanogaster* sperm swim backwards as a method of efficient transportation through the female reproductive tract (Kottgen et al., 2011). Although the mechanism for this backwards swimming behavior is unknown, it raises important questions both in regard to its physiological importance and the specific molecular targets within the sperm's axoneme that could lead to change in this behavior. It is feasible to hypothesize that insect sperm possesses the ability to swim backwards is likely an evolutionarily conserved mechanism and an alternative method to maneuvering through the female reproductive tract or method of fertilization. Future experiments may include calcium titrations along with manipulating phosphorylation levels and how those play a role in backwards motility, flagellar beating, and the ability of the sperm to swim backwards. Collectively, trypsin activation has been seen in both Hemi- and Holometabolous insects as an activator of the PAR leading to the activation of sperm motility.

In summary, calcium is a necessary second messenger for the activation and maintenance of mosquito sperm motility. Downstream protein phosphorylation also plays a role and an increased phosphorylated state in the presence of calcium can activate sperm while the absence of calcium leads to backwards motility. The mechanisms that control this behavior remains to be seen. The following chapters will discuss the potential mechanism(s) that mobilize calcium into the sperm following activation using proteomic

and pharmacological approaches and by specifically looking at changes in flagellar behavior under a variety of conditions.

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Figures and Tables

Conditions	*n*
Insect Ringer + Trypsin	24
Insect Ringer - Trypsin	3
CFR + EGTA + Accessory Glands	3
CFR + EGTA	3
CFR + EGTA + Trypsin	3
CFR + Trypsin + $CaCl_2$	3
Insect Ringer + Okadaic Acid	3
CFR + EGTA + Okadaic Acid	3

Table 2.1. Sperm motility in various environmental conditions. Working concentrations trypsin (2 µg/ml), EGTA (4 mM), $CaCl_2$ (5 mM), and okadaic acid (20 µM). CFR: Calcium-free Ringer.

Conditions	**Motile (%)**	**Nonmotile (%)**	**(*n*=)**
Insect Ringer + Trypsin	97	3	24
Insect Ringer - Trypsin	14	86	3
CFR + EGTA + Accessory Glands	1	99	3
CFR + EGTA	0	100	3
CFR + EGTA + Trypsin	18	82	3
CFR + EGTA + Trypsin + $CaCl_2$	91	9	3
Insect Ringer + Okadaic Acid	88	12	3
CFR + EGTA + Okadaic Acid	83	17	3

Table 2.2. The percentage of motile and immotile sperm in control assays using whole sperm incubated in varying solutions. Mature sperm were dissected out of the seminal vesicles and incubated for five minutes in different media. Unless otherwise stated, accessory glands and testes were removed during dissection. Working concentrations: trypsin (2 µg/ml), EGTA (4 mM), $CaCl_2$ (5 mM), and okadaic acid (20 µM). CFR: Calcium-free Ringer.

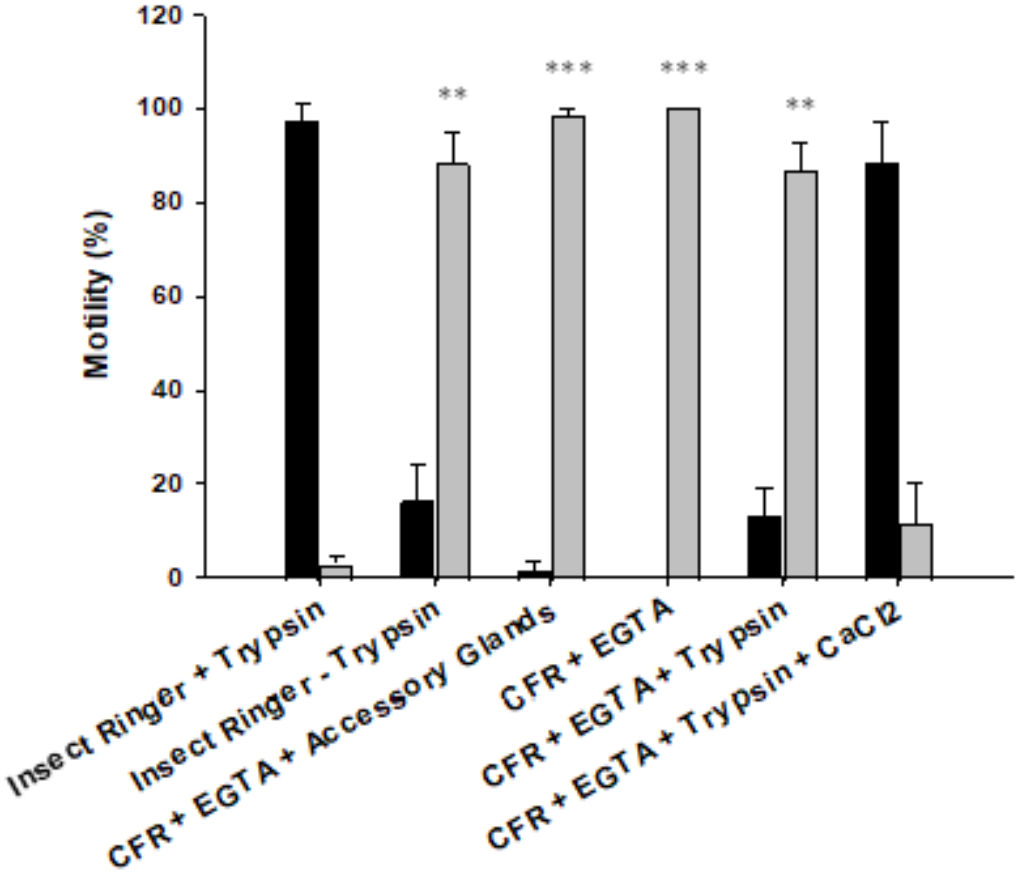

Figure 2.1. The percentage of motile and immotile sperm in control assays using whole sperm incubated in varying solutions. Motile sperm (black bars) and immotile sperm (gray bars). All bars represent averages with SEM bars. Using a t-test with equal variances not assumed p-value: *** 0.0001 to 0.001, ** 0.001 to 0.01, and ns ≥ 0.05.

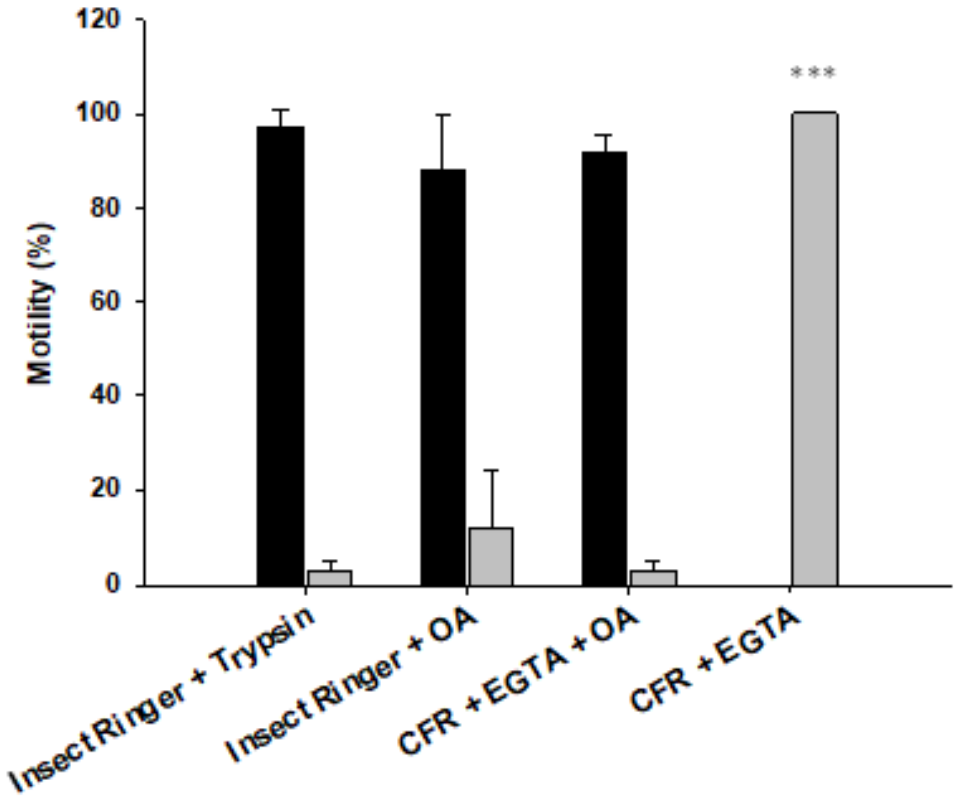

Figure 2.2. The percentage of motile and immotile sperm in the presence of increased phosphorylation. Motile sperm (black bars) and immotile sperm (gray bars). All bars are represented as averages with SEM bars. Using a t-test with equal variances not assumed p-value: *** 0.0001 to 0.001. CFR: Calcium-free Ringer, and OA: Okadaic acid.

Chapter 3: *Culex pipiens* Proteome and the Mechanisms Responsible for Mobilizing Calcium

Abstract

Various organisms including mammals and aquatic animals utilize and highly express CatSper, a pH sensitive and voltage-gated channel to mobilize the calcium proven to be necessary for sperm motility. The sperm of the *Culex* mosquito do not possess CatSper. Therefore, calcium mobilization must be regulated by a different protein. To determine which proteins, exist in the proteome of the *Culex* sperm, protein analyses were conducted which included samples from isolated mature sperm and testes. These results illustrated what proteins exist in the *Culex* sperm proteome. Along with identifying sperm component proteins, it aided in the detection of proteins involved in calcium mobilization and calcium homeostasis in *Culex* sperm. Following protein identification, sperm were incubated and treated with antagonists to determine the effect of the inhibition of intracellular calcium on sperm motility.

Introduction

Many animals use a microtubule-based flagellum to power sperm movements. The eukaryotic sperm flagellum is a highly complex structure consisting of hundreds of proteins used to generate and regulate motility with the microtubule-based structure, the axoneme, which is evolutionarily conserved across taxa (Inaba, 2011). The ability of the axoneme to bend is due to the sliding of axonemal doublet microtubules through the action of axonemal dynein located on the inner and outer arms of microtubule doublets (Gibbons 1981; Satir and Avolio 1986; Inaba, 2011). Although its precise mechanism is unclear, calcium likely plays a role in controlling kinase activity ultimately resulting in the phosphorylation of axonemal proteins leading to the activation and maintenance of flagellar beating and progressive motility.

There is substantial support for calcium's role as a second messenger in numerous physiological transformations in sperm that lead to successful fertilization in both invertebrates and vertebrates although the mechanisms for mobilizing intracellular calcium differ (Vines et al., 2002; Gao et al., 2003; Pitts et al. 2014; Endo et al., 2019; Hwang et al., 2019; Lissabet et al., 2019; Beltran et al., 2020). While the majority of research has focused on mammalian and marine invertebrate systems (Vines et al., 2002; Lissabet et al., 2019; Beltran et al., 2020), limited work on various insect sperm systems has also revealed that calcium plays a critical role in flagellar beating (Gao et al., 2003; Pitts et al. 2014).

Mammalian sperm utilize CatSper, a pH sensitive, voltage-gated calcium channel found exclusively in the flagellar sperm plasma membrane that is necessary for sperm

motility and successful fertilization (Ren et al., 2001; Carlson et al., 2003; Quill et al., 2003; Qi et al., 2007; Hwang et al., 2019). Numerous studies support a role for CatSper in successful fertilization since mice with compromised or inactive CatSper are infertile (Ren et al., 2001; Carlson et al., 2003; Quill et al., 2003; Qi et al., 2007; Hwang et al., 2019) likely because decreased calcium influx mediated by CatSper prevents the downstream activation of various components that stimulate capacitation, hypermotility, chemotaxis, and the acrosome reaction.

A mechanism utilized by one non-mammalian organism is the sodium/calcium exchanger. When herring sperm spawn, they are initially immotile until they are activated by the sperm motility initiating factor (SMIF), a glycoprotein localized to the micropylar region of the herring egg (Vines et al., 2002). Both extracellular sodium and extracellular calcium play a role in herring sperm motility and fertilization (Vines et al., 2002). When herring sperm were incubated in diluted sodium-free seawater in the absence of SMIF, they were still able to initiate motility, however motility was drastically reduced in 25 mM extracellular sodium and entirely inhibited in 150 mM (Vines et al., 2002). SMIF-induced sperm motility considerably decreased in the presence of 350 mM extracellular sodium and was fully inhibited by 500 mM (Vines et al., 2002). Both diluted sodium-free and SMIF-induced sperm motility required at least 1 mM extracellular calcium (Vines et al., 2002). The need of both a decrease in extracellular sodium and the presence of extracellular calcium indicates that a sodium/calcium exchanger may play a role during ligand-induced herring sperm motility activation (Vines et al., 2002). The SMIF-induced motility was blocked when specific calcium channel inhibitors were used (Vines et al.,

2002). Sperm treated with 10 µM flunarizine, a low affinity Ca^{2+} channel antagonist, displayed 98% motility inhibition while incubation with 10 µM nifedipine, an L-type channel blocker, displayed 90% motility inhibition (Vines et al., 2002). SMIF-induced motility was also inhibited when sodium/calcium exchanger inhibitors were applied (Vines et al., 2002). At a concentration of 10 µM, KB-R7942 displayed 99% motility inhibition, dichlorbenzamil displayed 97% motility inhibition, and bepridil 96% displayed motility inhibition (Vines et al., 2002). Further, when SMIF was added to sperm in seawater, motility was initiated along with an increase in intracellular calcium and a decrease in intracellular sodium (as detected by fluorescent intracellular probes) (Vines et al., 2002).

The SMIF stimulated increase in intracellular calcium was inhibited by various calcium channel blockers. At 100 µM nifedipine, sperm displayed 90% motility inhibition; at 10 µM flunarizine, dichlorobanzamil, or KB-R7943, sperm displayed 98%, 97%, and 99% motility inhibition, respectively, and bepridil treated sperm displayed 96% motility inhibition at 50 µM (Vines et al., 2002). The sodium efflux was completely inhibited by bepridil and partially inhibited by flunarizine at 20 µM (Vines et al., 2002). These data suggest that the glycoprotein SMIF stimulates a sodium/calcium exchanger running in reverse with a calcium influx and sodium efflux (Vines et al., 2002). This mechanism likely evolved whereby herring sperm remain immotile in sea water until contact is made with the appropriate ligand on the egg (Vines et al., 2002).

Voltage-gated calcium channels play a key role in managing calcium influx in cells that is initiated by a depolarization of the plasma membrane leading to the

stimulation of an array of physiological responses (Lissabet et al., 2019). Sperm samples were taken from male Atlantic salmon, *Salmo salar*, and were treated with verapamil, a specific voltage-gated L-type calcium channel blocker at concentrations of 0.5 mM and 1.0 mM (Lissabet et al., 2019). Verapamil had previously been shown to block voltage-gated calcium channels in other aquatic organisms leading to reduced sperm motility (Lissabet et al., 2019). When sperm of *S. salar* were treated with verapamil, there was a significant decrease in the total progressive motility (Lissabet et al., 2019) suggesting that a voltage-gated L-type calcium channel may play a role in the initiation of sperm motility in *S. salar* (Lissabet et al., 2019).

The role of a voltage-gated T-type channel was also investigated in *S. salar* (Beltran et al., 2020). Sperm samples were collected from adult male *S. salar* and were treated with NNC-55-0396, a highly selective voltage-gated T-type calcium channel antagonist, at the concentrations 0.5 µM and 2.0 µM (Beltran et al., 2020). Sperm incubated in the presence of NNC-55-0396 for one hour resulted in a significant reduction of both progressive and total motility at both concentrations (Beltran et al., 2020). These results suggest that a voltage-gated T-type calcium channel is present in these sperm and that it plays a role in sperm motility (Beltran et al., 2020). It has also been argued that CatSper is a necessary channel for sperm motility in the Atlantic salmon, and that collectively, these data contribute evidence for the importance of voltage-gated channels in the activation and maintenance of sperm motility (Beltran et al., 2020).

During internal fertilization in the Japanese red bellied newt, *Cynops pyrrhogaster,* motility and the acrosome reaction are stimulated by the outer egg-jelly surface (JE) which contains ligands that induce the acrosome (known as acrosome reaction-inducing substance, (ARIS) and sperm motility-initiating substances (SMIS) (Endo et al., 2019). Both ARIS and SMIS are necessary for successful fertilization (Endo et al., 2019). During fertilization, intracellular calcium increases twices, the first time in the presence of ARIS during the acrosome reaction and the second time in the presence of SMIS during sperm motility initiation (Endo et al., 2019).

As sperm normally possess many calcium channels, determining those that play a role in ARIS and SMIS signaling pathways are vital for understanding signaling pathways that are involved in reproductive success. The N-methyl D-aspartate type glutamate receptor (NMDAR) is an ionotropic receptor that leads to an influx in calcium through the binding of glutamate and the release of a magnesium block (Endo et al., 2019). The NMDAR was confirmed present in *C. pyrrhogaster* sperm through RNA-sequencing of the testis and confirmed through RT-PCR (Endo et al., 2019). When sperm were incubated in the presence of JE and the antagonists memantine and MK-801, at concentrations of 1 mM and 500 µM, respectively, the acrosome reaction was inhibited (Endo et al., 2019). When sperm were incubated in salt solution, they displayed a spontaneous acrosome reaction that was not inhibited in the presence of memantine but successfully inhibited by 500 µM MK-801 (Endo et al., 2019). These data indicate that the NMDAR is a facilitator of both the ARIS-induced and spontaneous acrosome reactions (Endo et al., 2019).

Progressive motility in the presence of SMIS was also inhibited by 10-100 µM memantine and 500 µM MK-801 implying that the NMDAR also mediates forward motility initiated by SMIS (Endo et al., 2019). When sperm were incubated in salt solution alone, the sperm failed to progress forward but did exhibit flagellar beating (Endo e al., 2019). Undulating membranes were inhibited by 100 µM memantine and 500 µM MK-801 and rescued by 1 mM NMDA, an agonist of the NMDAR (Endo et al., 2019). NMDA, however, did not stimulate forward progressive motility in sperm (Endo et al., 2019). These data suggest that the NMDAR is necessary to facilitate the acrosome reaction and forward motility through its assist in the two-phase elevation of intracellular calcium (Endo et al., 2019). It also indicates that the NMDAR facilitates spontaneous undulating of the membrane (Endo et al., 2019).

Although limited work has been done with insect sperm, many mechanisms involving specific proteins have been implicated in modulating calcium. The PKD2 channel, a family of calcium-activated cation channels, was the first protein shown to play a role in directional movement of sperm inside the female reproductive tract (Gao et al., 2003). PKD2 is abundantly expressed in *Drosophila melanogaster* larval and adult testes and localizes to the sperm flagellum, the head, and the acrosome (Gao et al., 2003). Gao et al. (2003) performed a targeted knockout consisting of homologous recombination between a mutated PKD2 donor sequence and the endogenous PKD2 locus (Gao et al., 2003). The knockout resulted in alleles that produced undetectable wild-type PKD2. When homozygous, the flies are viable but with significant loss-of-function mutations (Gao et al., 2003). The viable males possessing these alleles still developed a normal

testis, but fertility was dramatically lowered (Gao et al., 2003). The F1 progeny produced from a cross with a mutant homozygous male and a wild-type female decreased their average number from 60 to 3 (Gao et al., 2003). When one copy of the wild-type transgene was introduced back into the flies, fertility increased to 50% suggesting that fully deactivating PKD2 leads to a decrease in male fertility (Gao et al., 2003).

To examine the effects on male fertility further, a sperm tail associated GFP reporter was used to quantify the amount of sperm produced in mutant males by analyzing the seminal vesicles (Gao et al., 2003). The size of the seminal vesicles in mutant males were indistinguishable from wild-type males and during mating, the sperm were found in the uterus of the female (Gao et al., 2003). Although not as many sperm were able to access the egg (79% in wild-type versus 3% in mutants) the egg hatching rate was normal in wild-type at 78.7% while affected in mutants at 2.7% (Gao et al., 2003).

In general, a wild-type male ejaculates ca. 4,000-6,000 sperm (Gao et al., 2003). During the mating period, a gelatinous plug forms in the female reproductive tract to prevent sperm from leaking. Immediately following ejaculation, wild-type sperm will migrate away from the gelatinous mating plug into the anterior uterus and into the spermathecae (Gao et al., 2003). This movement of the sperm is likely regulated by directional cues (Gao et al., 2003). Wild-type sperm possess sperm that move quickly into the uterus and by the end of mating, sperm migration to the anterior uterus and into the spermathecae has been successfully completed (Gao et al., 2003). This differs significantly from the sperm of mutant males (Gao et al., 2003). Their sperm move

substantially slower and have reduced flagellar beating, they do not migrate to the anterior uterus, nor do they move into the spermathecae (Gao et al., 2003). This result suggests conflict with directional cues as well as an inability of sperm to be stored in storage organs in mutant male sperm (Gao et al., 2003). These data indicate that PKD2 mediates calcium influx at various points along the sperm flagellum and is responsible for initiating flagellar beating leading to downstream sperm movement in the uterus and migration to the spermathecae (Gao et al., 2003).

Another protein suggested to play a role in calcium regulation in insect sperm is the odorant receptor coreceptor (Orco). Insects possess olfactory receptors that are heteromeric, ligand gated, seven transmembrane ion channels that normally comprise an odorant specific binding subunit (OR) and a coreceptor subunit (Orco) (Guo et al., 2018). Together they form the complex Or/Orco, a cation channel that allows an influx of calcium (Guo et al., 2018). However, Jones et al. (2011) discovered that the Orco subunit alone can form a functional cation channel without the presence of the OR subunit and could be gated by the agonist VUAA1 (Jones et al., 2018). Orco is made up of seven membrane-traversing helical segments (S1-S7) with an intracellular amino terminus and an extracellular carboxy terminus (Butterwick et al., 2018). As a complex, Orco forms a tetrameric protein with four subunits surrounding a pore (Butterwick et al., 2018).

Pitts et al. (2014) discovered the existence of Orco in the sperm flagella of one mosquito species, *Anopheles gambiae*, and RNA sequencing was used to quantify transcript numbers in *An. gambiae* testis (Pitts et al., 2014). An Orco antibody was utilized to investigate the protein expression of *An. gambiae* Orco (AgOrco) in the testes

and found that the AgOrco protein is expressed in male sperm and is localized along the flagella of fully developed sperm (Pitts et al. 2014). In support of AgOrco's role in sperm motility, flagellar beating significantly increased when AgOrco was in the presence of two Orco agonists, VUAA1 (the agonist that could be a potential gate for Orco) and VUAA4 (Pitts, et al., 2014). However, in the presence of the AgOrco antagonist, VU0183254, flagellar beating did not occur (Pitts et al., 2014). When the agonists were introduced along with the antagonist, the activation of flagellar beating was severely lowered (Pitts et al., 2014). To further investigate the roles of agonists on flagellar activation and beating, Pitts et al. (2014) used an Orco mutant strain of *Aedes aegypti* (Pitts et al., 2014). When the wild-type strain was presented with an agonist, there was a strong response while Orco mutants were unresponsive (Pitts et al., 2014).

This study has taken a closer look into the proteome of the full sperm and testes of the *Culex* mosquito and has, for the first time isolated and identified both detergent-soluble and -insoluble proteins. Once potential calcium regulators were identified, they were manipulated with agonists or antagonists in order to identify their role in general beat frequency and progressive motility in mosquito sperm.

Materials and Methods

Chemicals and Reagents

Trypsin, EGTA, EDTA, $NiCl_2$, $ZnCl_2$, Verapamil, 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), amantadine, memantine, ifenprodil, D-AP5 were purchased through Sigma Aldrich (Burlington, MA). NNC 55-0395 dihydrochloride was purchased through Tocris Bioscience (Bristol, UK). 8-Br-cADPR and 8-Br-N1-cIDPR were purchased through Axxora Life Sciences (Farmingdale, NY). L-cis-diltiazem was purchased from Abcam (Cambridge, UK).

Insect Sperm Media

Insect Ringer contained 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 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. Calcium Free Insect Ringer (CFR) contained 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, 4 mM EGTA, pH 7.2. Phosphate Free Insect Ringer (PFR) contained 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 1 mM glucose, 20 mM HEPES, pH 7.2. Insect Ringer that was free of phosphates and sulfates (PSFR) contained 110 mM NaCl, 7 mM KCL, 2.4 mM $MgCl_2$, 1.2 mM $NaHCO_3$, 1 mM glucose, 20 mM HEPES, 4 mM EGTA, pH 7.2. All of the Ringers were compared to each other to confirm there were no statistically significant differences between them (Figure 3.1).

Mosquitoes and Dissections

The *Culex pipiens* wild-type line was maintained at 25°C, 80% relative humidity, 16:8 light/dark cycle and given sugar cubes as a sugar source. The *C. pipiens* line is autogenous, meaning a blood meal is not required for the females to lay egg rafts, therefore only sugar was provided for the females. Egg rafts were collected in trays of recycled larval water and were hatched in cups containing deionized water where they were then placed into larger pans. The larvae fed on a 1:1 diet of mouse food and brewer's yeast with additional fish oil. The adult mosquitoes were collected using a battery powered aspirator and were anesthetized and sorted using CO_2. Males were then placed individually in 1.25-ounce Solo cups (product #P125, Solo Cup Company) with lids (product #PL1, Solo Cup Company). Chloroform-soaked cotton balls were introduced to the cups as a method of sacrificing the mosquitoes. The male reproductive tract was then dissected out in phosphate buffered saline (PBS): 20 mM Na_2HPO_4, 100 mM Na_2HPO_4 and placed on a clean glass side with Insect Ringer (recipes below). All sperm used were mature sperm collected from the seminal vesicles. Unless otherwise stated, testes and accessory glands were removed prior to the assay leaving only the seminal vesicles.

To test general sperm motility under various conditions, the sperm were placed in a total of 20 µl of varying Insect Ringers. Trypsin unless otherwise stated was used in each condition and had a working concentration of 2 µg/ml. The seminal vesicles were opened on the slide in Insect Ringer to release the sperm. A coverslip with clay at each corner was added to the top of the slide and pressed down until the coverslip touched the

liquid. This allowed the sperm to move freely in three dimensions. The slide was then moved to a microscope stage and the behavior was monitored on a upright darkfield microscope (Nikon Labophot, Tokyo, Japan) with a high-speed camera (Edgertronic, SP1, Campbell, CA).

Sperm in the control assays (Table 3.1) were monitored every to two-minutes over the course of twenty minutes in various Insect Ringers. Behavior was recorded for 10 seconds at 100 frames/second. During the 10 second recordings, the microscope stage was shifted to three different fields of view to account for overall sperm behavior in the solution. These behavioral recordings were then analyzed using image processing software (ImageJ, NIH). This process was repeated for each biological replicate. Sperm in the experimental treatment assays were monitored every two-minutes over the course of 20 minutes in either Insect Ringer, CFR, PFR, or PSFR containing a channel blocker (Table 3.1)**.** At each timepoint, the behavior was recorded for 10 seconds at 100 frames/second. During the 10 second recordings, the microscope stage was shifted to three different fields of view to account for overall sperm behavior in the solution. These behavioral recordings were then analyzed using image processing software (ImageJ). This process was repeated for each biological replicate. The number of total sperm analyzed in both the control and treatment assays ranged from ~150-400.

RNA Isolation and cDNA Synthesis

RNA isolation was performed on approximately 200 *C. pipiens* testes which were homogenized in TRIzol™ reagent (Invitrogen, Carlsbad, CA). RNA was extracted using the Zymo Direct-zol RNA Miniprep Plus (Zymo Research, Irvine, CA, catalog number: R2073) according to the manufacturer's protocol. Concentrations of RNA were determined using a NanoDrop™ 2000c Spectrophotometer (Thermo Scientific, Waltham, MA).

Aliquots of 100 ng testis RNA were converted to cDNA using the Zymo-Seq RiboFree Universal cDNA Kit (Zymo Research, catalog number: R3001) with the temperature and cycle times provided in the manufacturer's protocol.

PCR Analysis

Primers were designed using the NCBI-Primer BLAST software. Primers were obtained from Integrated DNA Technologies and 10 µM working solutions were diluted from a 100 µM primer stock. The working primers are listed in (Table 3.2). cDNA PCR products were separated on 1.5% agarose gels in 0.5x TBE using a Bio-Rad horizontal gel rig at 100V for 60 min. PCR products were visualized by ethidium bromide staining. Standard PCR reactions of 50 µL using MyTaq HS Red (Bioline, Memphis, TN), 1 µL of each primer, a minimum of 100 ng of RNA, and RNase/DNase free water were conducted using the following conditions: initial denaturation at 95 °C for 1 min, followed by 35 cycles of 95 °C for 15 sec, 55 °C for 15 sec, 72 °C for 10 sec, and a final extension at 72 °C for 5 min.

Silver Staining of SDS-PAGE Gels

Following separation of protein samples by SDS-PAGE, the gel was placed directly into fixative (200 ml ddH_2O, 200 ml methanol, 400 µl 37% formaldehyde) and incubated overnight with gentle shaking. Following the overnight incubation, the gel was removed from the fix and washed for 30 minutes (500 ml ddH_2O, 2.5 mg DTT) with gentle shaking. The wash was then removed, and silver stain was added (300 ml ddH_2O, 0.3g $AgNO_3$) with five minutes of gentle shaking and then placed directly on a light box for 20 minutes. The silver stain was then removed and washed briefly with ddH_2O. The gel was then rinsed twice briefly with developer (400 ml ddH_2O, 12 g Na_2CO_3, 200 µl 37% formaldehyde) and then incubated in developer until bands appeared. Citric acid (9.66g citric acid brought up to 20ml with ddH_2O) was then added when the stain had reached a peak development. The gel was incubated in the citric acid for ten minutes. Following the citric acid incubation, the gel was then incubated and washed in ddH_2O for one hour before photographs were taken of the gel.

Protein Isolation

For isolation of sperm membrane proteins, 100 seminal vesicles were squeezed (releasing the mature sperm) into buffer containing 50 mM Tris (pH 7.5) and 1 mM EDTA along with a protease inhibitor cocktail consisting of: 1 mM PMSF, 104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin. The sperm suspension was centrifuged in a microfuge at 1,000 rpm for 15 minutes to collect large tissue debris. This procedure only pellets large tissue debris,

leaving the sperm in the supernatant. The supernatant was collected, and 0.1% CHAPS was added 1:1 to the supernatant. The sperm were treated with detergent for one hour on ice with intermittent vortexing. The sperm were then centrifuged at 14,000 rpm for 30 minutes in a microfuge and supernatant containing solubilized membrane proteins were collected. The remaining pellet containing insoluble proteins was resuspended in buffer containing 50 mM Tris (pH 7.5) and 1 mM EDTA along with the same protease inhibitor cocktail as listed above. Samples were stored at -20°C and sent to the University of California, San Diego Department of Chemistry and Biochemistry Proteomics Core to be processed.

Protein Profile

Whole sperm protein profile samples consisted of 75 whole seminal vesicles squeezed into sample buffer 50 mM Tris (pH 7.5) and 1 mM EDTA containing protease cocktail consisting of: 1 mM PMSF, 104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, and 1.5 mM pepstatin. and 1 mM PMSF. Detergent-insoluble and -soluble protein fraction profile samples consisted of 75 seminal vesicles squeezed into Tris/EDTA buffer containing protease inhibitors spun in a microfuge at 1,000 rpm for 15 minutes to collect miscellaneous tissue. The supernatant was collected and treated 1:1 with 0.1% CHAPS. The sample was kept on ice and vortexed every 10 minutes for one hour. The sample was then spun in a microfuge at 14,000 rpm for 30 minutes. The whole sperm, detergent-soluble supernatant and pellet were run on an SDS-PAGE.

Proteome Assembly

As the seminal vesicles are squeezed into buffer in order to remove the sperm, it is plausible that proteins from the seminal vesicles may have contaminated the sample. Because these proteins would likely be in low abundance, the criteria used in order to reject these proteins by mass spectrophotometer was to only include proteins that are represented by two or more peptides at >95% confidence throughout the sample. This then only leaves proteins that are in high abundance and that are definite components of the *Culex* sperm proteome.

Statistical Analysis

All data was analyzed using the statistical software SigmaPlot 14.5. Welch's t-tests were used to compare the data between two groups, the control and one treatment at a time. Equal variances were not assumed. P-values: **** < 0.0001, *** 0.0001 to 0.001, ** 0.001 to 0.01, * 0.01 to 0.05, and ns ≥ 0.05. T_{50} were determined using sigmoidal dose dependent or linear regression best fit curves.

Results

Part I: Proteomic Analysis

Previous work characterizing *Culex* sperm motility demonstrated that extracellular calcium was required for initiation of flagellar beating and forward progressive motility. These data imply that sperm possess a mechanism for regulation of calcium entry. To identify potential regulators of calcium entry, proteomic analyses of testis, whole sperm, and detergent-soluble (i.e., membrane) proteins, as well as detergent-insoluble proteins, was conducted. A previously assembled whole sperm proteome (Thaler et al., *submitted*) was examined as well. A few potential calcium regulatory candidates were discovered in the whole sperm and testis proteomes. To enrich for membrane bound receptors that may have a reduced abundance that precludes detection in the whole sperm proteome, a detergent-soluble fraction of sperm proteins was collected and analyzed by mass spectrometry. The detergent-insoluble material from this preparation was analyzed as well. These varied approaches were used to give the most comprehensive list of the potential calcium regulators present in the *Culex* sperm (accession numbers located in Table 3.3).

Testis

The analysis of the *Culex* testis resulted in the identification of 1,551 unique proteins including a voltage-gated anion selective channel (VDAC) that may play a role in the facilitation of intracellular calcium as reported in other studies (Shoshan-Barmatz et al., 2017), and an NMDA-regulated protein 1 which may be linked to the N-methyl D-

aspartate receptor (NMDAR) (Table 3.3). The NMDAR is an ionotropic receptor that, when activated, results in calcium influx of calcium in *Cynops pyrrhogaster* sperm (Endo et al., 2019). Although the NMDA-regulated protein 1 is present in the proteome, it is uncertain as to whether the NMDA receptor (NMDAR) is present. Therefore prior to any future manipulation of the receptor, cDNA synthesis, PCR, and gel electrophoresis were used to confirm the existence of the NMDAR. Both the VDAC and NMDAR proteins were considered as potential calcium regulators.

Whole Sperm

The analysis of the *Culex* whole sperm proteome resulted in the identification of 950 unique proteins. Examination of this proteome revealed that mosquito sperm possess several potential proteins that may play a role in regulating calcium mobilization (Table 3.3). These include the voltage-gated anion selective channel (VDAC), an odorant receptor, and a sodium/calcium exchanger. Studies have demonstrated that VDAC is highly calcium permeable and plays an important role in calcium homeostasis by facilitating calcium into and out of mitochondria (Shoshan-Barmatz et al., 2017). An odorant receptor was also present and flagged as a potential calcium mediator in the proteome since the odorant receptor co-receptor (Orco) has been observed in the flagella from another mosquito taxon, *An. gambiae*. Although Orco normally heterodimerizes with an odorant receptor forming a complex, studies have indicated that the Orco subunit can form a functional cation channel without the presence of an odorant receptor (Pitts et al., 2011; Guo et al., 2018). Similarly, proteomic studies revealed that Orco was also

detected in whole *Culex* sperm, although in very low abundance (Thaler et al., submitted). Of significance, the odorant receptor protein detected in this most recent proteome was not identified as Orco and a protein BLAST analysis indicated that it is a more general odorant protein (24a), a chemoreceptor that responds to volatile chemicals as seen in various insects (Brand et al., 2018). Additionally, only one peptide of the odorant protein 24a was detected, which eliminates it from the list of significant components of this *Culex* sperm proteome sample. The sodium/calcium exchanger was also present in the proteome and studies have demonstrated that while the exchanger normally functions to remove calcium from the cell, when depolarized it can function in reverse, supplying the cell with calcium (Vines et al., 2002). As with the odorant receptor, the sodium/calcium exchanger, was in very low abundance, less than two peptides, and was disregarded as a significant component of the *Culex* sperm proteome as well.

Following identification of potential calcium regulating proteins, sperm membranes were analyzed more directly using a detergent isolation procedure to enrich for membrane components. This report is the first to describe isolation of membrane proteins from an insect sperm.

Detergent-Insoluble Proteins

Following disruption of the lipid bilayer of whole sperm using the zwitterionic detergent CHAPS, detergent-soluble proteins were collected in the supernatant and were anticipated to consist of transmembrane proteins, while the detergent-insoluble proteins

pelleted during centrifugation. However, while analyzing the detergent-insoluble protein fraction, a likely calcium regulating candidate was revealed.

The analysis of the *Culex* sperm detergent-insoluble protein fraction, resulted in the identification of 238 unique proteins. The insoluble fraction contained several expected insoluble proteins including an abundance of α-tubulin, which can be seen in high frequency at approximately 50 kDa (Figure 3.2). The detergent-insoluble protein fraction also included a voltage-gated anion selective channel (VDAC), a voltage-dependent T-type calcium channel subunit alpha-1G, and a cyclic nucleotide gated cation channel (Table 3.3). As previously mentioned, VDAC may function as an intracellular calcium regulator (Shoshan-Barmatz et al., 2017). The voltage-gated T-type calcium channel has been studied in *S. salar* sperm and has been observed as playing a significant role in the regulation of calcium (Beltran et al., 2020). A cyclic nucleotide gated cation channel has been identified in sea urchin sperm; however, it specifically facilitates a potassium efflux (Kaupp et al., 2008). Although the cyclic nucleotide gated cation channel detected here did not specify the specific cation that it regulated, it was still considered as a potential calcium channel.

Detergent-Soluble Membrane Proteins

The analysis of the *Culex* sperm detergent-soluble membrane protein fraction resulted in the identification of 199 unique proteins, of which only the ryanodine receptor plays a potential role in the mobilization of calcium in the sperm (Table 3.3). The ryanodine receptor regulates intracellular calcium as a transmembrane protein located on

the smooth sarcoplasmic and endoplasmic reticulum. The ryanodine receptor has also been shown, in some cells, to localize to the plasma membrane and to regulate and mediate calcium entry across the plasma membrane (Rosker et al., 2009). The detergent-soluble protein data also consists of various cotransporters, receptors, and channels; however, none are potential calcium regulating candidates.

Protein profile

The protein profiles of whole mature sperm were compared to that of the detergent-soluble and -insoluble sperm protein fractions, using SDS-PAGE (Figure 3.2). As expected, the total protein profile contained the greatest abundance and diversity of proteins while the insoluble protein fraction included a subset of those proteins and the membrane-soluble protein fraction possessed the fewest proteins. These data were consistent with and supported by the data received from the proteomic sequencing.

Additional Analyses to Detect Orco and NMDAR

Two potential candidates of interest, Orco and the NMDAR, could not be unequivocally detected in the proteomic analyses we conducted. Therefore, we used a direct PCR analysis of testis cDNA to evaluate their presence in the testis and possible status as candidates for calcium entry in sperm.

Sperm isolated from the testis, suppress transcriptional activity because of the organization of chromatin during spermiogenesis resulting in an inability to isolate RNA directly from sperm. Therefore, RNA was isolated from testis from recently eclosed

males as the young testis would possess transcripts used to produce the sperm. cDNA was synthesized from the RNA and amplified using RT-PCR.

Although the NMDA-regulated protein 1 was detected in the whole sperm proteome sample generated in this study, it is uncertain as to whether the actual NMDA receptor is present. Therefore, primers were designed using the mRNA of the NMDAR subunit 1 which is a subunit that exists in all isoforms of the protein. After the cDNA was run on an agarose gel, the NMDAR subunit 1 amplicon was present at the correct size, 300 bp (Figure 3.3).

Primers were designed using Orco mRNA. The cDNA was run on an agarose gel and an amplicon was present at the correct molecular weight, 639 bp (Figure 3.4).

An Orco antibody was obtained (gifted from Lawrence Zweibel, Vanderbilt University) to further test the presence of Orco in the *Culex* sperm. An SDS-PAGE protein gel was run using male heads as a positive control, ovaries as a negative control, whole sperm, and testis. Although Orco was present in *Culex* sperm heads and testis, it was not detected in sperm. There are many reasons why Orco was not detected in *Culex* sperm. It is likely that the Orco peptide abundance was at a level that it was too low to be revealed by mass spectrometry or Western Blot and only able to be detected by amplified cDNA, it is not located on the sperm, or the antibody does not recognize the isoform in *Culex* sperm (Western Blot not shown).

Analysis of T-Type Voltage-Gated Calcium Channel

To confirm the presence of a T-type voltage- gated calcium channel, primers were designed using T-type voltage-gated calcium channel mRNA. The cDNA was run on an agarose gel and an amplicon was present at the correct molecular weight of 432 bp (Figure 3.5).

Calcium Regulating Candidates

Based on the combined findings of the proteomic studies in *Culex* sperm and membrane fractions and the PCR studies on expressed genes from testis only a few candidates were considered viable as potentially significant in regulating intracellular calcium. modulators of calcium flux in sperm. These included a voltage-dependent anion selective channel, the NMDA receptor, the ryanodine receptor, a cyclic-nucleotide-gated cation channel, and a voltage-dependent T-type voltage-gated calcium channel.

II. Motility Analysis

After analyzing the *Culex* proteome and determining the potential candidates that regulate calcium in the sperm, a number of motility assays were performed. These assays included the use of specific agonists and antagonists that would alter the normal function of the protein in question. Sperm that were activated by a protease in the presence of these antagonists were observed under dark field optics using a high-speed camera every two minutes starting at two minutes over a twenty-minute period. Sperm in the presence of an agonist were not activated by protease but were observed under the same

conditions. A disruption of the protein that provides calcium entry to the sperm following protease activation should result in a decrease in sperm motility. Similarly, activation of a protein that facilitates calcium entry into the sperm in the absence of the protease should stimulate sperm motility. Deviations in general sperm motility (motile versus immotile) were observed and in some cases forward progressive motility was examined. Motility scoring parameters for each experimental treatment are explained in the following sections.

Voltage Dependent Anion Selective Channel (VDAC)

As a VDAC was in very high abundance in three out of the four proteomic samples, 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS), an anion dependent voltage-gated channel inhibitor was applied to the sperm and the effects on sperm motility recorded. The sperm were incubated in Insect Ringer containing calcium, trypsin, and 1 mM DIDS (IC_{50}: 1-10 µM in human HEK 293T cells (Costa et al., 2018) (Figure 3.6). An IC_{50} is a measurement that specifies how much of an inhibitory substance is needed to inhibit an event *in vitro* (e.g., flagellar motion or progressive motility). At ten minutes, there was a reduction sperm motility (85% to 61%), but this change was not significant relative to controls. It was not until 16 minutes that the sperm reached a significantly inhibited state (28%). The T_{50} (the time it takes for 50% of the sperm to become immotile) for the control and VDAC was 23.38 minutes and 13.79 minutes respectively with a statistically significant difference (p-value = 0.007). These

data demonstrate an inhibitory effect on the sperm however the voltage-dependent anion selective channel is unlikely candidate for facilitating calcium entry in sperm.

L-Type Voltage-Gated Channel

Voltage-gated channels were in high abundance within the proteomic sequencing data. Therefore, a voltage-gated L-type channel inhibitor was used to determine its possible role in calcium entry that regulates sperm motility. Verapamil, a potent voltage-gated L-type calcium channel blocker (Bkaily and Jacques, 2009), was used on activated mosquito sperm and the effect on their motility was observed (IC_{50}: 143 nM in human native heart cells (Zhang et al., 1999)). The sperm were incubated in Insect Ringer containing calcium, trypsin, and 1 mM verapamil (Figure 3.7). At twelve minutes there was a slight, but not significant, reduction in motility (93% to 87%) with a T_{50} for the control and verapamil of 36.73 and 25.11 minutes respectively with no statistically significant difference (p-value = 0.484). These data infers that an L-type voltage-gated channel is unlikely involved in the facilitation of calcium entry in sperm involved in sperm motility.

NMDA Receptor

Based on the proteome sequencing results, an NMDA-receptor regulated protein is present in the *Culex* mosquito proteome. Primers designed from the NMDAR subunit 1 confirmed the presence of the NMDAR subunit 1 in the testes of the *Culex* sperm. Channel blockers and allosteric inhibitors specific for the NMDAR were examined on the

sperm and general sperm behavior was observed. The sperm were incubated in Insect Ringer containing calcium, trypsin, and either 1 mM of the channel blockers amantadine (IC_{50}: 40 µM in human HEK 293T cells (Blanpied et al., 2005)) or memantine (IC_{50}: 1 µM in human tsA201 cells (Glasgow et al., 2017)), or 1 mM of the allosteric inhibitors ifenprodil (IC_{50}: 0.5 to 10 µM in rodent cerebellum (Carter et al., 1988)) or D-AP5 (IC_{50}: 1 µM in rodent spinal neurons (Lodge et al., 1988)).

In the presence of the channel blocker amantadine, the motility was slightly reduced at twelve minutes, to 58%, but for the most part remained similar to the motility in the control for the remaining minutes suggesting that amantadine does not have a strong inhibitory effect on the sperm motility (Figure 3.8). Memantine displayed similar uninhibited motility with 57% of the sperm displaying motility at twelve minutes similar to the control (Figure 3.8). The T_{50} for the control, amantadine, and memantine were 19.01, 16.42, and 14.74 minutes respectively with no statistically significant difference (p-value for amantadine and memantine = 0.503 and 0.230 respectively). These data indicates that amantadine and memantine are ineffective inhibitors.

The allosteric inhibitors ifenprodil and D-AP5 were also investigated as inhibitors of the NMDAR, and when comparing the response at twelve minutes (similarly to the channel blockers), ifenprodil displayed uninhibited motility with 83% of sperm possessing flagellar beating (Figure 3.9). D-AP5 also demonstrated uninhibited sperm motility of 83% at twelve minutes (Figure 3.9). As the sperm motility was not inhibited even at high concentrations of channel blockers and allosteric inhibitors, these data suggest that the NMDAR is not a facilitator of calcium entry in the sperm. These

allosteric inhibitors had a T_{50} for the control, ifenprodil, and D-AP5 of 23.71, 21.07, and 21.93 respectively with no statistically significant difference (p-value for ifenprodil and D-AP5 = 0.869 and 0.317 respectively). These results suggest that ifenprodil and D-AP5 are ineffective as inhibitors of sperm motility.

Ryanodine Receptor

The ryanodine receptor was also present as a potential facilitator of calcium entry in sperm. The ryanodine receptor is located on the smooth endoplasmic reticulum of cells and is generally responsible for the release of calcium from intracellular stores (Laver 2007). Both an antagonist, 8-Br-cADPR (IC_{50}: 1 µM in rodent NG108-15 cells (Bowden et al., 1999)), and an agonist, 8-Br-N1-cIDPR, of the ryanodine receptor, were used in separate experiments to test the role of the receptor in sperm motility. Sperm were incubated in Insect Ringer containing calcium, trypsin, and 1 mM 8-Br-cADPR (Figure 3.10). In the presence of the antagonist 8-Br-cADPR, there was no significant difference in sperm motility over the course of twenty minutes with a T_{50} for the control and 8-Br-cADPR of 27.04 and 28.63 minutes, respectively (p-value = 0.616). Sperm were then incubated in the presence of Calcium-free Ringer, no trypsin, 4 mM EGTA and 1 mM of the agonist 8-Br-N1-cIDPR (Figure 3.11). If the ryanodine played a role in mobilizing intracellular calcium utilized in motility, sperm would be activated even without trypsin and calcium in the presence of the agonist. Over the course of twenty minutes only at two minutes was there a significant difference and the sperm motility did not otherwise deviate from the control. As the sperm are being activated and not inhibited, a T_{50} could

not be calculated. The lack of inhibition and activation by 8-Br-cADPR and 8-Br-N1-cIDPR, respectively, makes the ryanodine receptor an unlikely mediator of calcium entry in sperm.

Cyclic Nucleotide Gated Cation Channel

The effect of a cyclic nucleotide gated cation channel activity on sperm motility was tested using the inhibitor L-*cis*-diltiazem (IC_{50}: 5 µM in salamander cone photoreceptors (Hart et al., 2003)). The sperm were incubated in Insect Ringer containing calcium, trypsin, and 1 mM L-*cis*-diltiazem. The overall motility was unaffected by L-*cis*-diltiazem and mimicked the flagellar frequency of the control (Figure 3.12). The T_{50} for the control and L-*cis*-diltiazem is 49.91 and 66.96 minutes respectively with no statistically significant difference (p-value = 0.813). Therefore, this demonstrates that L-*cis*-diltiazem is an unsuccessful inhibitor and indicates that the cyclic nucleotide gated channel is not facilitating calcium entry necessary for sperm motility.

T-Type Voltage-Gated Calcium Channel

$NiCl_2$

Ni^{2+}, a divalent metal cation, has been demonstrated as a successful antagonist against T-type voltage-gated cation channels (Zamponi et al., 1996). The effect of $NiCl_2$ on sperm motility was tested by incubating sperm in Insect Ringer containing calcium, trypsin, and 1 mM $NiCl_2$ (IC_{50}: 10 µM in rodent thalamocortical relay neurons (Kim et al., 2001)). At eight minutes there was an observed decline in the percent of motile sperm

(81% to 46%) and by twelve minutes, the percent of motile sperm was reduced even further (46% to 11%) with a T_{50} for the control and $NiCl_2$ of 17.38 and 7.97 minutes with no statistically significant different (p-value = 0.1) (Figure 3.13). Given the substantial decline in motility by 12 minutes, various concentrations of $NiCl_2$ on the sperm were examined at this time point (Figure 3.14a). Videos of the sperm at twelve minutes were scored for general motility.

There was a detectable reduction in overall sperm motility by $NiCl_2$ in a concentration dependent manner. At concentration of 1 µM, 10 µM, and 20 µM, the sperm displayed uninhibited and robust motility. As the concentration approached 50 µM, motility was reduced by 50% and at a concentration of 1 mM, only 10% of sperm were motile. To further confirm 1 mM $NiCl_2$ has an inhibitory, but not lethal, effect, sperm were incubated in CFR, 1 mM $NiCl_2$, 4 mM ethylenediaminetetraacetic acid (EDTA, a chelator that preferentially binds divalent metal ions), and 2 mM $CaCl_2$. Over the twenty-minute observation period (Figure 3.15), sperm motility was similar to control motility and did not show any inhibition. When the $NiCl_2$ was removed, sperm motility decreased over the twenty minutes likely due to the EDTA chelating the calcium preventing a strong calcium influx in the sperm. When EDTA was removed, sperm motility recovered, and the sperm displayed uninhibited motility. The T_{50} for $NiCl_2$ + EDTA (control), no $NiCl_2$ + EDTA, and no $NiCl_2$ – EDTA were 29.97, 13.22, 28.84 minutes respectively with the only statistically significant difference occurring between $NiCl_2$ + EDTA (control) and no $NiCl_2$ + EDTA (p-value = 0.007). There was no

statistically significant difference between $NiCl_2$ + EDTA (control) and no $NiCl_2$ – EDTA (p-value = 0.492).

$ZnCl_2$

Zn^{2+} is also known to block voltage-gated channels (Busselberg et al., 1992) and was introduced to the sperm in a manner similar to $NiCl_2$. The sperm were incubated in phosphate-free Insect Ringer containing calcium, trypsin, and 1 mM $ZnCl_2$ (IC_{50}: 0.8 µM in human tsA201 cells (Traboulsie et al., 2006)). At the twelve-minute mark, there was a slight decrease in the sperm motility to 68% with the T_{50} for the control and $ZnCl_2$ of 21.21 and 14.07 minutes respectively with no statistically significant difference (p-value = 0.268) (Figure 3.13). Subsequently, a series of increasing concentrations of $ZnCl_2$ were tested and sperm motility at twelve minutes scored to confirm the effects on motility (Figure 3.14b). In the presence of 100 µM $ZnCl_2$, there was a slight reduction in sperm motility.

Forward Progressive Motility in $NiCl_2$ and $ZnCl_2$

After examining the effects of $NiCl_2$ and $ZnCl_2$ on general sperm motility, forward progressive motility in the presence of each compound was examined (Figure 3.16). $NiCl_2$ mirrored the initial general motility timeline results with a significant reduction in the number of sperm displaying progressive motility at ten minutes and almost no forward progression from twelve minutes to twenty minutes. The T_{50} for the control and $NiCl_2$ were 21.29 and 8.95 minutes respectively with no statistically significant difference (p-value = 0.06). $ZnCl_2$ initially displayed an insignificant effect on

the general motility (scored as the presence of flagellar beating) of the sperm; however, when forward progression was quantified, $ZnCl_2$ displayed a significant reduction in the number of sperm exhibiting forward progressive motility over the course of twenty minutes. The T_{50} for the control and $ZnCl_2$ were 22.63 and 0 minutes respectively displaying a statistically significant difference (p-value = 0.04).

The dose dependence of this inhibition of forward progressive motility was examined at twelve minutes for both $ZnCl_2$ (IC_{50}: 0.8 µM in human tsA201 cells (Traboulsie et al., 2006)) and $NiCl_2$ (IC_{50}: 10 µM in rodent thalamocortical relay neurons (Perez-Reyes, 2003)). As the concentration of $NiCl_2$ increased, the forward progression of the sperm became strongly inhibited (Figure 3.17a). At a concentration of 1 mM $NiCl_2$ there was complete inhibition of forward progression. Inhibitory effects of $NiCl_2$ still remained at 10 µM, but the sperm regained forward progression at 1 µM.

When forward progression of the sperm was scored in the presence of $ZnCl_2$, there was a marked effect on the sperm. (Figure 3.17b). The inhibition of forward progression was present at concentrations as low as 1 µM and as the concentration of $ZnCl_2$ increased, the forward progression of the sperm continued to remain strongly suppressed and at 1 mM forward progression was completely inhibited. When forward progression was evaluated the T_{50} of $NiCl_2$ and $ZnCl_2$ were eight minutes and two minutes respectively illustrating the effectiveness of these inhibitors on sperm locomotion.

These data suggests that a voltage-gated T-type calcium channel may be responsible for mobilizing calcium leading to sperm activation and progressive forward motility.

NNC 55-0396 Dihydrochloride

Sperm motility was significantly affected by the presence of $NiCl_2$ and $ZnCl_2$. To further support a T-type voltage-gated calcium channel as the mechanism responsible for facilitating calcium entry, the effect of a highly selective T-type voltage-gated calcium channel inhibitor, NNC 55-0396 dihydrochloride (NNC) ($IC_{50:}$ 7 µM in human HEK 293T cells (Huang et al., 2004)), was tested. When sperm were incubated in the presence of 50 µM NNC, only 68% of the sperm were motile by two minutes, and the percentage of motile sperm decreased further (to 26%) by four minutes. Sperm motility was completely inhibited by six minutes or longer. As the timepoint with the greatest magnitude of difference for the inhibition of the sperm compared to the control is at eight minutes, various concentrations of NNC were tested on the sperm at this timepoint in order to observe overall effectiveness on sperm motility (Figure 3.18). with the T_{50} of the control and NNC were 25.15 and 3.79 minutes respectively with a statistically significant difference (p-value = 0.22).

There was a clear reduction in overall sperm motility in the presence of NNC and as the concentration of NNC increased, the inhibitory effect on sperm motility also strongly increased in a concentration dependent manner (Figure 3.19).

Discussion

As seen in various non-mammalian organisms, including two insect species, calcium is necessary for sperm motility and is mobilized by sperm through by a variety of proteins including PKD2 (Gao et al., 2003), reverse sodium/calcium exchangers (Vines et al., 2002), the N-methyl D-glutamate cation channel (Endo et al., 2019), and L- and T-type voltage-gated channels (Bkaily and Jacques 2009; Beltran et al., 2020). These data suggest that in the absence of CatSper, other proteins likely play a role in sperm in mobilizing the calcium in insects and other lower invertebrates.

Following proteomic sequencing of *Culex* sperm, there was a clear picture as to what proteins exist in the proteome. After narrowing down the proteins that were associated with calcium mobilization, specific agonists and antagonists were used to test the role of each of these channels. Applying agonists and antagonists that inhibited VDAC, L-type voltage-gated channels, NMDAR channels, ryanodine receptors, and cyclic nucleotide gated cation channels showed no significant disruption of sperm motility. In contrast, the T-type voltage-gated channel inhibitor $NiCl_2$ produced a strong inhibition of general motility and together $NiCl_2$ and $ZnCl_2$ created a significant inhibition in forward progression of the sperm. Since $NiCl_2$ and $ZnCl_2$ are robust T-type voltage-gated channel inhibitors (Busselberg et al., 1992; Zamponi et al., 1996), it is likely that a T-type voltage-gated channel is responsible for mobilizing calcium in sperm.

The highly selective T-type voltage-gated calcium channel inhibitor NNC 55-0396 dihydrochloride was used to independently assess the role of a T-type channel in sperm motility. It strongly inhibited general motility and forward progressive motility.

These data strongly imply that a T-type voltage-gated calcium channel is the facilitator of the calcium that is necessary for the maintenance of motility in sperm. Voltage-gated calcium channels are important transducers of membrane potential changes into intracellular calcium signals that start various physiological events (Catterall 2011). Calcium channels in various cell types activate through membrane depolarizations and facilitate calcium influxes in response to these depolarizations and action potentials (Catterall 2011). Calcium entering the cell through these voltage-gated calcium channels result in the initiation of many cellular pathways (Catterall 2011). There are many members of the voltage-gated calcium channel family which fall into either high- and low-voltage activated channels (Catterall 2011). High-voltage activated channels include L-, P/Q-, N-, and R- type while low-voltage activated channels include T-type (Catterall 2011). The T-type voltage-gated calcium channel found in the *Culex* sperm activate at small and very negative depolarizations, inactivate quickly, and deactivate slowly while high-voltage activated channels require much larger and less negative membrane potentials (Perez-Reyes, 2003; Catterall, 2011). Calcium influxes through these low voltage T-type voltage-gated calcium channels stimulate low-threshold spikes which lead to bursts of action potentials (Perez-Reyes, 2003). These T-type channels recover quickly from inactivation which allows them to contribute to these bursts (Perez-Reyes, 2003). These characteristics result in rapid sperm activation and continuous motility in the presence of constant calcium influxes. It also allows for swift directional changes from forward swimming sperm to backwards swimming sperm in the presence of increased phosphorylation. CatSper is also a low voltage gated calcium channel, it however as the

protein is most closely related to a transient receptor potential channel is unclear as to whether the channel possess the ability to have action potential bursts (Sun et al., 2017).

Following these data, localizing the T-type voltage-gated calcium channel using an appropriate antibody and immunofluorescence would assist in understanding its distribution along the length of the sperm and give an idea as to how abundant the protein is on the sperm. Furthermore, to extend our knowledge of this channel and the role it plays in sperm motility, genetic modifications and targeted mutagenesis may be applied. Genome editing techniques include transcription activator-like effector nuclease (TALENs), zinc finger nuclease, or through the CRISPR-Cas9 system. Genetic knockout or genetic silencing of the gene(s) responsible for the calcium mobilizing protein needed for sperm motility may result in inhibited sperm locomotion and additional confirmation that the T-type voltage-gated channel plays a necessary role in the regulation and facilitation of calcium needed for sperm motility.

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Figures and Tables

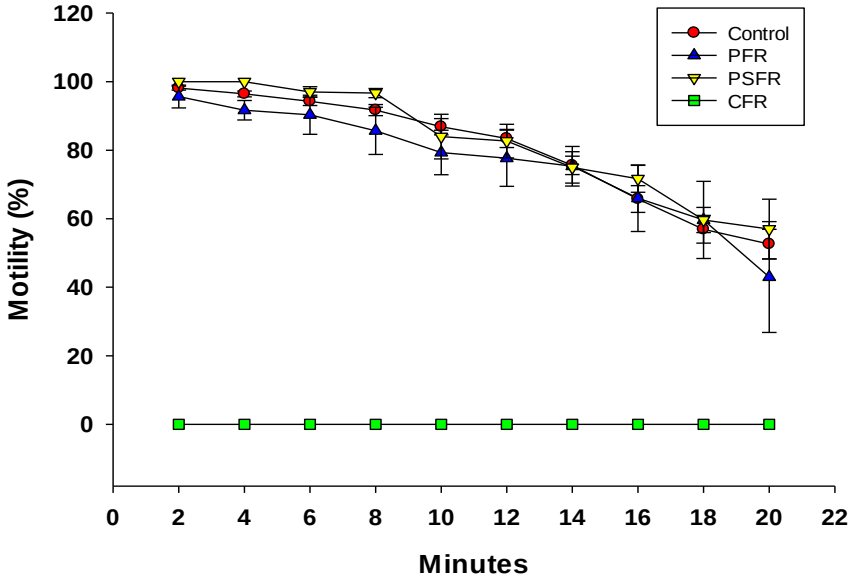


Figure 3.1. Averaged controls of mosquito sperm motility using various Insect Ringer solutions. Modified Ringer solutions were manipulated in several ways to successfully manage various compounds. The control, phosphate-free Ringer. and phosphate- and sulfate-free Ringer T_{50} were 23.95 minutes, 21.21, and 23.38 minutes. Respectively. with no significant statistical differences. Data represent averages and error bars are SEM values, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer, PFR: Phosphate-free Ringer, PSFR: Phosphate- and Sulfate-free Ringer.

Conditions	***(n=)***
Insect Ringer + Trypsin (Control)	30
PFR + Trypsin (Control)	3
PSFR + Trypsin (Control)	3
CFR + Trypsin (Control)	3
Insect Ringer + Trypsin + 1 mM $NiCl_2$	3
PFR + Trypsin + 1 mM $ZnCl_2$	3
Insect Ringer + Trypsin + 1 mM Verapamil	3
PSFR + Trypsin + 1 mM DIDS	3
Insect Ringer + Trypsin + 50 µM NNC 55-0396 Dihydrochloride	3
Insect Ringer + Trypsin + 1 mM Amantadine	3
Insect Ringer + Trypsin + 1 mM Memantine	3
Insect Ringer + Trypsin + 1 mM Ifenprodil	3
Insect Ringer + Trypsin + 1 mM D-AP5	3
Insect Ringer + Trypsin + 1 mM 8-Br-cADPR	3
Insect Ringer + Trypsin + 1 mM 8-Br-N1-cIDPR	3
Insect Ringer + Trypsin + 1 mM L-cis-Diltiazem	3

Table 3.1. Summary of various conditions for motility assays. The working trypsin concentration was 2 ug/ml. CFR: Calcium-free Ringer, PFR: Phosphate-free Ringer, PSFR: Phosphate- and Sulfate-free Ringer, DIDS: 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid.

Target	Forward	Reverse	Product Size (bp)
NMDAR SB 1	ATGGAGAATTT GACCTGCGC	TGCCAACGTAA CTGCATCTG	300
Or7 (Orco)	CAAGCTGTACT CGTGGTGGA	GAGTTGGGTCG GTAGGTGTC	639
T-type VGCC	CCTTGCGGGAA TTGCGAAAA	CAACCACGTTC AAGCTGTCG	432
Actin (Control)	TTCAACTCGCC AGCCATGTA	TTTGTGGCGTT GTTTGGTTTG	787

Table 3.2. Working primers used in PCR analysis.

A

Gene ID	Annotation	Peptide #
CPIJ000967	Voltage-dependent anion-selective channel (282 aa)	34

B

Gene ID	Annotation	Peptide #
CPIJ000967	Voltage-dependent anion-selective channel (282 aa)	33
CPIJ003426	NMDA receptor-regulated protein 1 (879 aa)	11

C

Gene ID	Annotation	Peptide #
CPIJ000967	Voltage-dependent anion-selective channel (282 aa)	7
CPIJ006595	Cyclic-nucleotide-gated cation channel (334 aa)	3
CPIJ018672	Voltage-dependent T-type calcium channel subunit alpha-1G (1258 aa)	2

D

Gene ID	Annotation	Peptide #
CPIJ001302	Ryanodine receptor (1339 aa)	2

Table 3.3. List of proteins that may play a role in facilitating calcium influx in *Culex* sperm. A) Whole sperm proteins. **B)** Testes protein. **C)** Detergent-insoluble protein fraction. **D)** Detergent-soluble protein fraction.

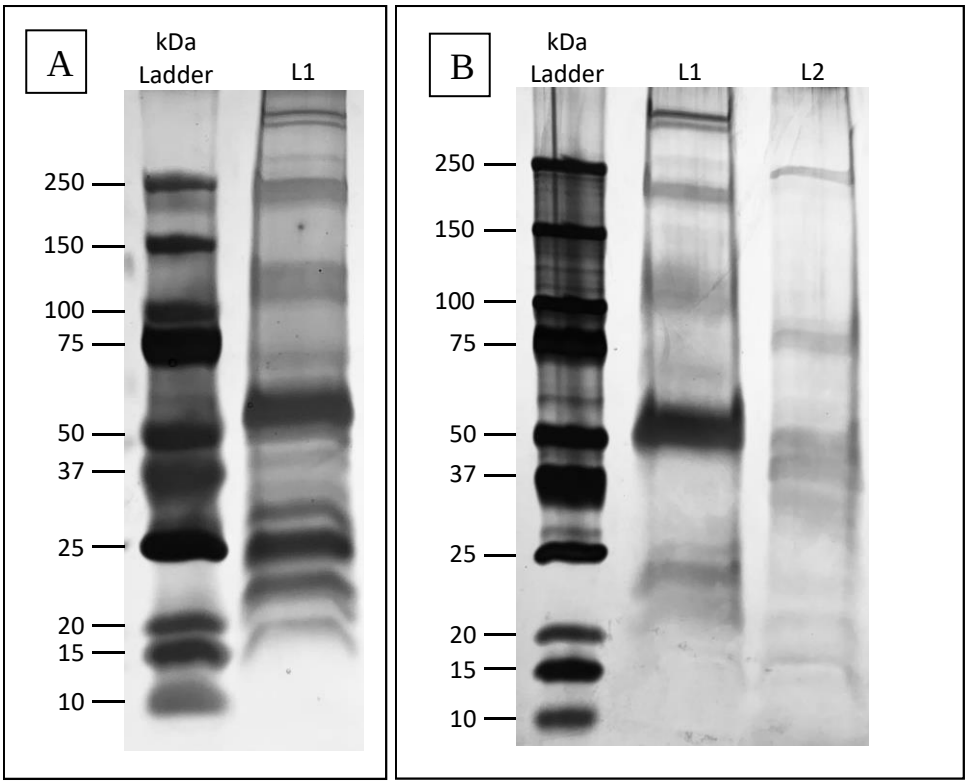


Figure 3.2. Protein profiles in isolated mature sperm. Proteins were run on an SDS-PAGE and stained using silver stain. **A)** Lane 1 represents whole sperm total protein profile. **B)** Lane 1 represents whole sperm insoluble protein profile and lane 2 represents whole sperm soluble protein profile.

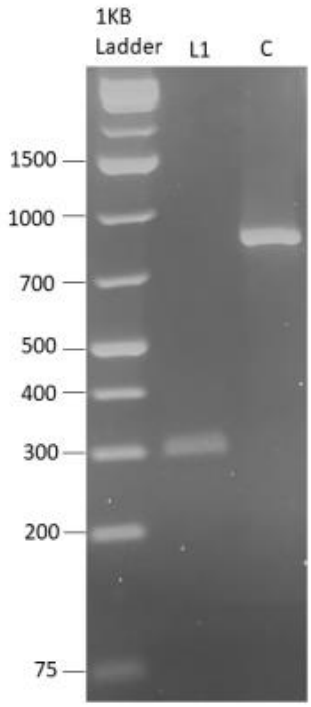


Figure 3.3. NMDAR subunit 1 PCR product run on an agarose gel. Lane 1 consists of NMDA subunit 1 PCR product run on a 1.5% agarose gel, product size is 300bp. Lane 2 (labeled C) is an actin control, product size 787bp.

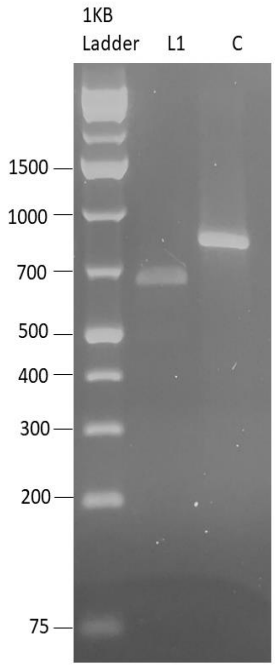


Figure 3.4. Or7 PCR product run on an agarose gel. Lane 1 consists of Or7 PCR product run on a 1.5% agarose gel, product size is 639 bp. Lane 2 (labeled C) is an actin control, product size 787 bp.

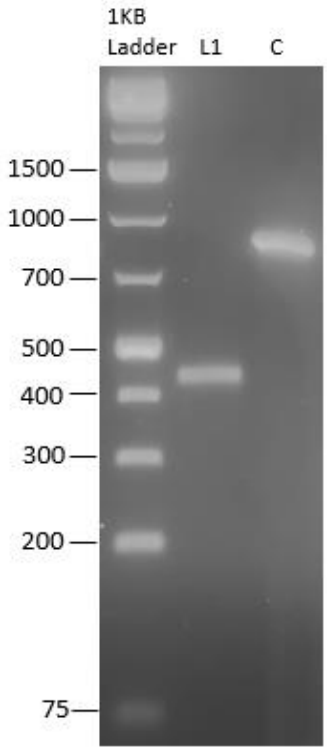


Figure 3.5. T-type voltage-gated calcium channel PCR product run on an agarose gel. Lane 1 consists of T-type voltage-gated calcium channel PCR product run on a 1.5% agarose gel, product size is 432bp. Lane 2 (labeled C) is an actin control, product size 787bp.

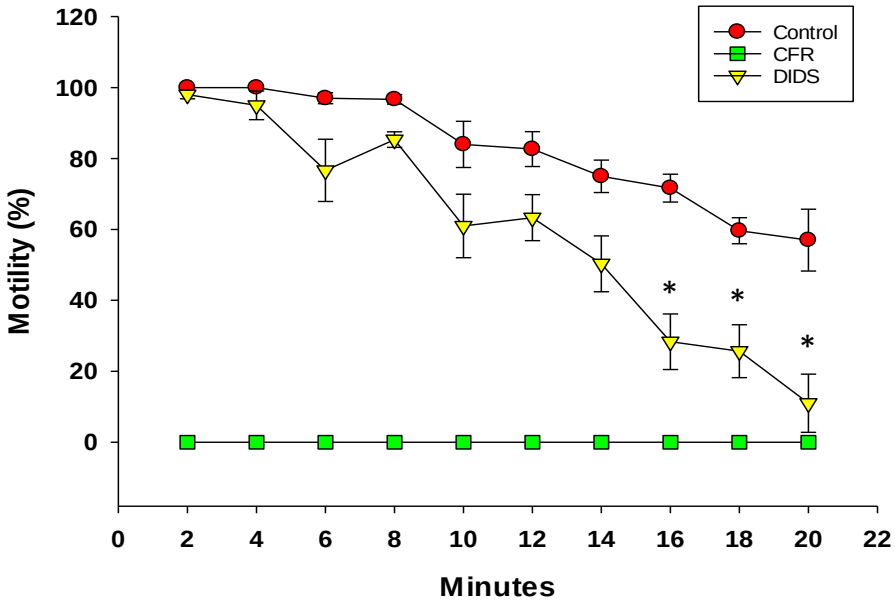


Figure 3.6. Percentage of sperm motility in the presence of the VDAC inhibitor, DIDS. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The NMDAR blockers amantadine and memantine had little effect on motility and only after 12 minutes incubation was there a slight, but significant, decrease in motility relative to the positive and negative controls (trypsin and CFR respectively). The control and DIDS T_{50} were 23.38 minutes and 13.79 minutes respectively with a significant statistical difference. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test between control and each treatment, with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

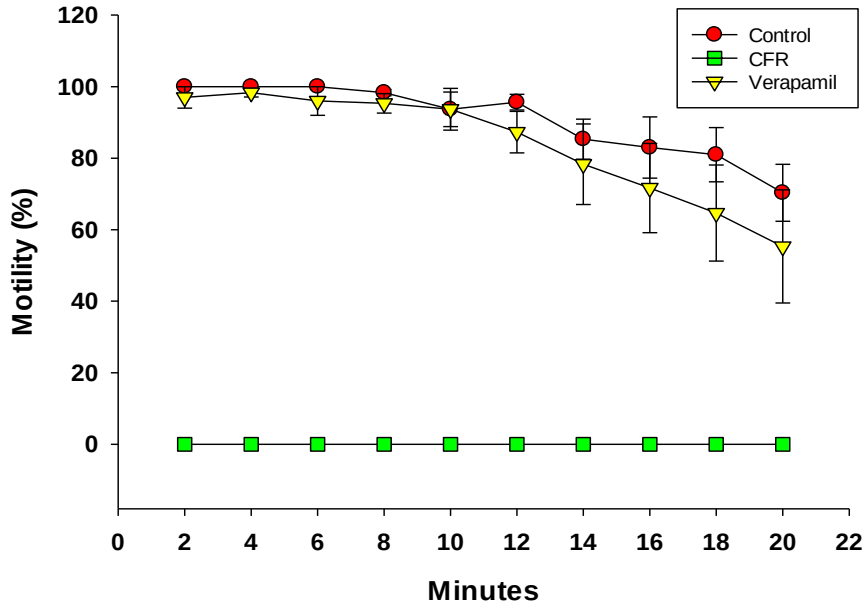


Figure 3.7. Sperm motility in the presence of the L-type voltage-gated channel inhibitor, verapamil. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The L-type voltage-gated channel inhibitor, verapamil had no effect on motility relative to the positive and negative controls (trypsin and CFR respectively). The control and verapamil T_{50} were 36.73 minutes and 25.11 minutes respectively with no significant statistical difference. Data shown are means ± SEM, (*n* = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

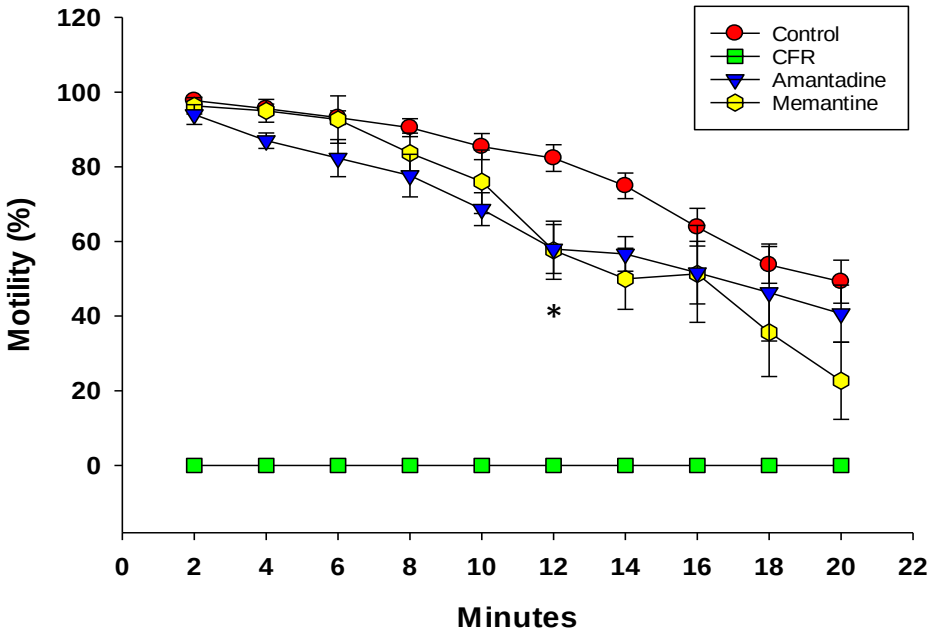


Figure 3.8. Sperm motility in the presence of the NMDAR channel blockers, amantadine and memantine. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The NMDAR blockers amantadine and memantine had little effect on motility and only after 12 minutes incubation was there a slight, but significant, decrease in motility relative to the positive and negative controls (trypsin and CFR respectively). The control, amantadine, and memantine T_{50} were 19.01 minutes, 16.42 minutes, and 14.74 minutes respectively with no significant statistical differences. Data shown are means ± SEM, (n = 3 at each time point). A t-test with equal variances not assumed was used for all comparisons. p-value: * 0.01 to 0.05; ns ≥ 0.05. CFR: Calcium-free Ringer.

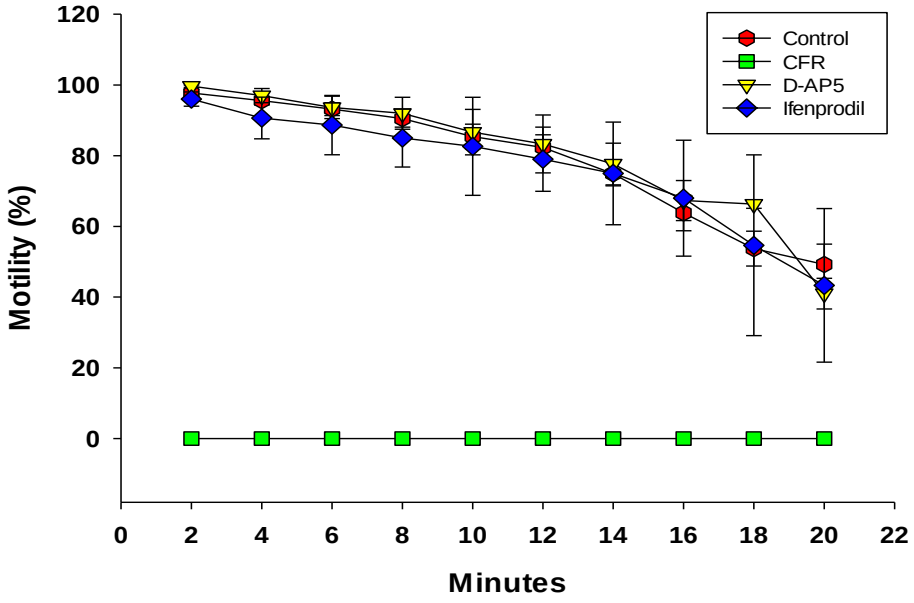


Figure 3.9. Sperm motility in the presence of NMDAR allosteric inhibitors, D-AP5 an ifenprodil. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The NMDAR allosteric inhibitors D-AP5 and ifenprodil had no effect on motility relative to the positive and negative controls (trypsin and CFR respectively). The control, ifenprodil, and D-AP5 T_{50} were 23.71 minutes, 21.07 minutes, and 21.93 minutes respectively with no significant statistical differences. Data shown are means ± SEM bars, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

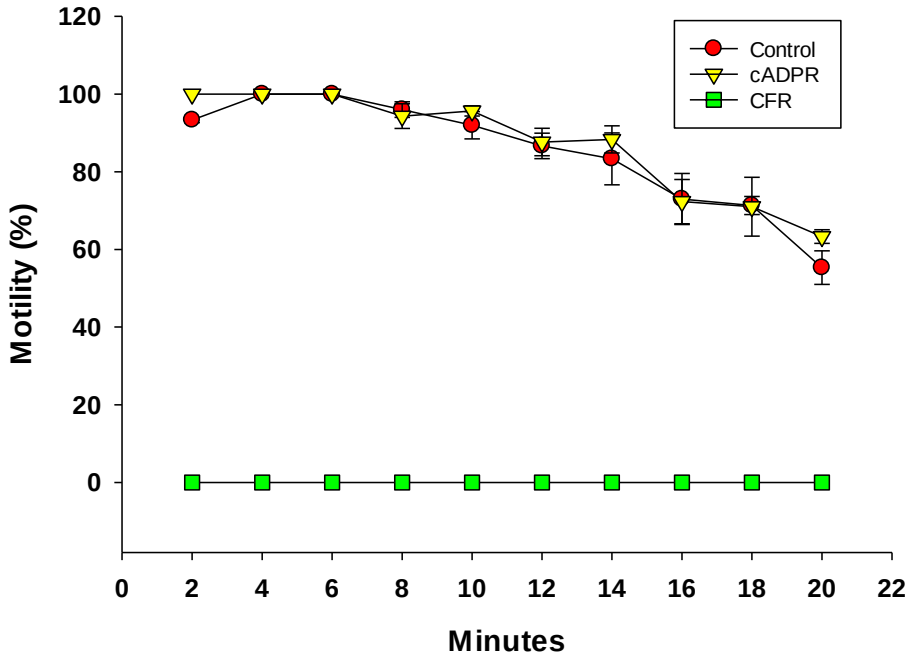


Figure 3.10. Sperm motility in the presence of the ryanodine receptor antagonist, 8-Br-cADPR. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The ryanodine receptor antagonist 8-Br-cADPR had no effect on motility relative to the positive and negative controls (trypsin and CFR respectively). The control and 8-Br-cADPR T_{50} were 27.04 minutes and 28.63 minutes respectively with no significant statistical difference. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

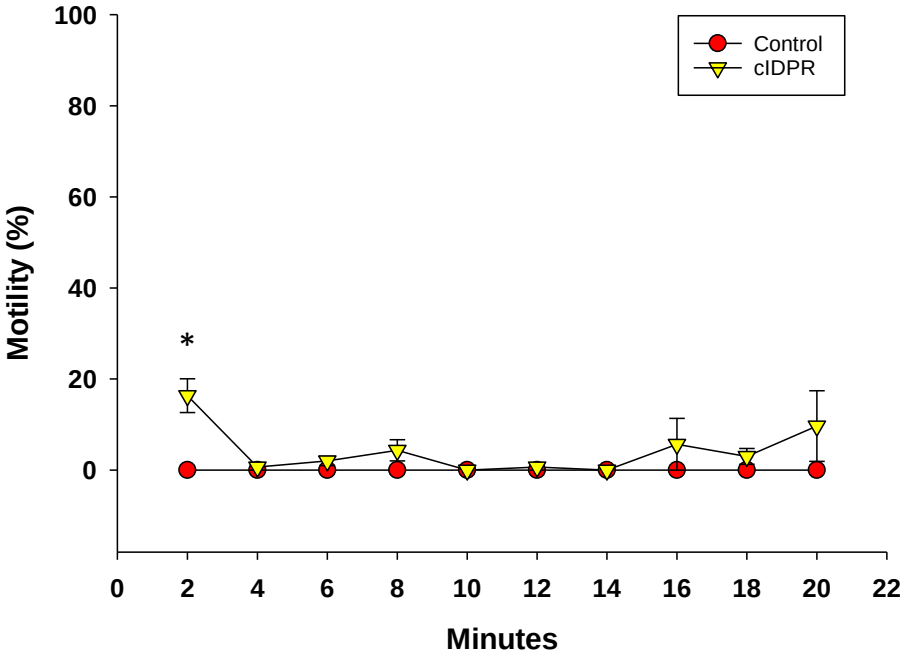


Figure 3.11. Sperm motility in the presence of the ryanodine receptor agonist 8-Br-N1-cIDPR. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The ryanodine receptor agonist 8-Br-N1-cIDPR had little effect on motility and only at two minutes incubation was there a slight, but significant, increase in motility relative to the positive and negative controls (trypsin and CFR respectively). As 8-Br-N1-cIDPR is used to activate instead of inhibiting, a T_{50} could not be calculated. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

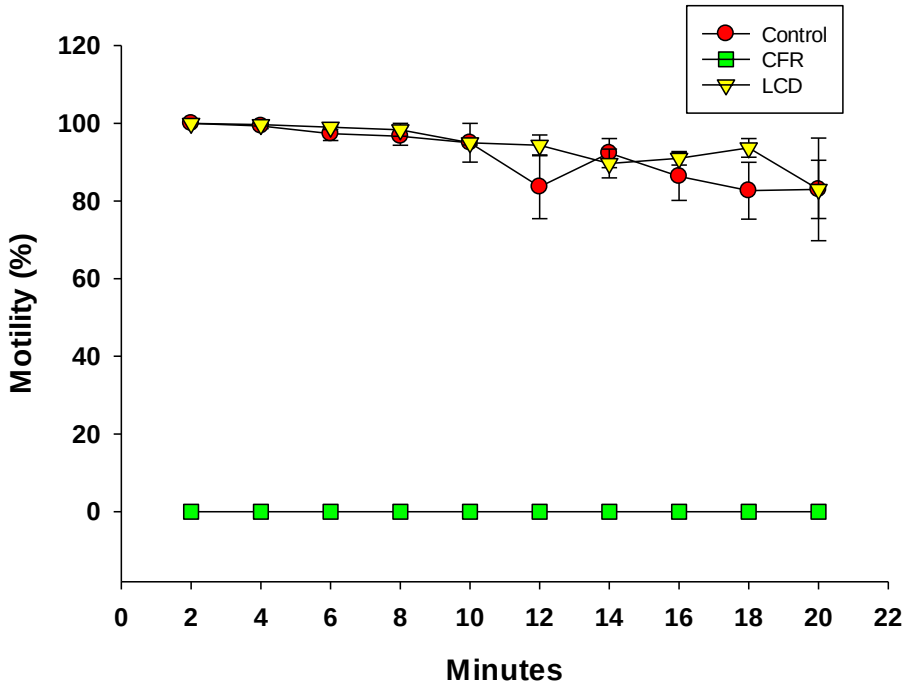


Figure 3.12. Sperm motility in the presence of the cyclic nucleotide gated cation channel inhibitor, L-*cis*-diltiazem. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The cyclic nucleotide gated cation channel inhibitor L-*cis*-diltiazem had no effect on motility relative to the positive and negative controls (trypsin and CFR respectively). The control and L-*cis*-diltiazem T_{50} were 49.91 minutes and 66.96 minutes respectively with no significant statistical difference. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer and LCD: L-*cis*-diltiazem.

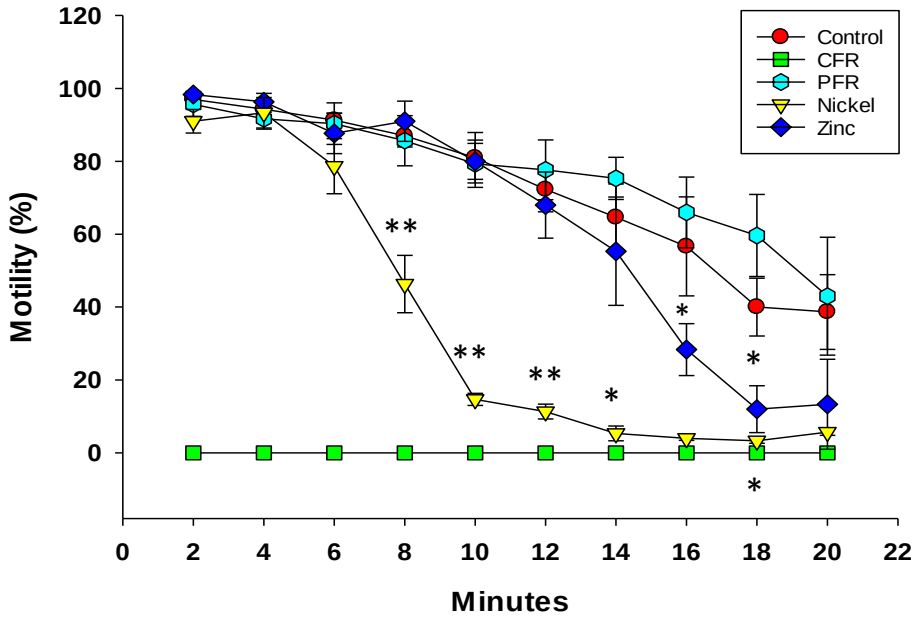
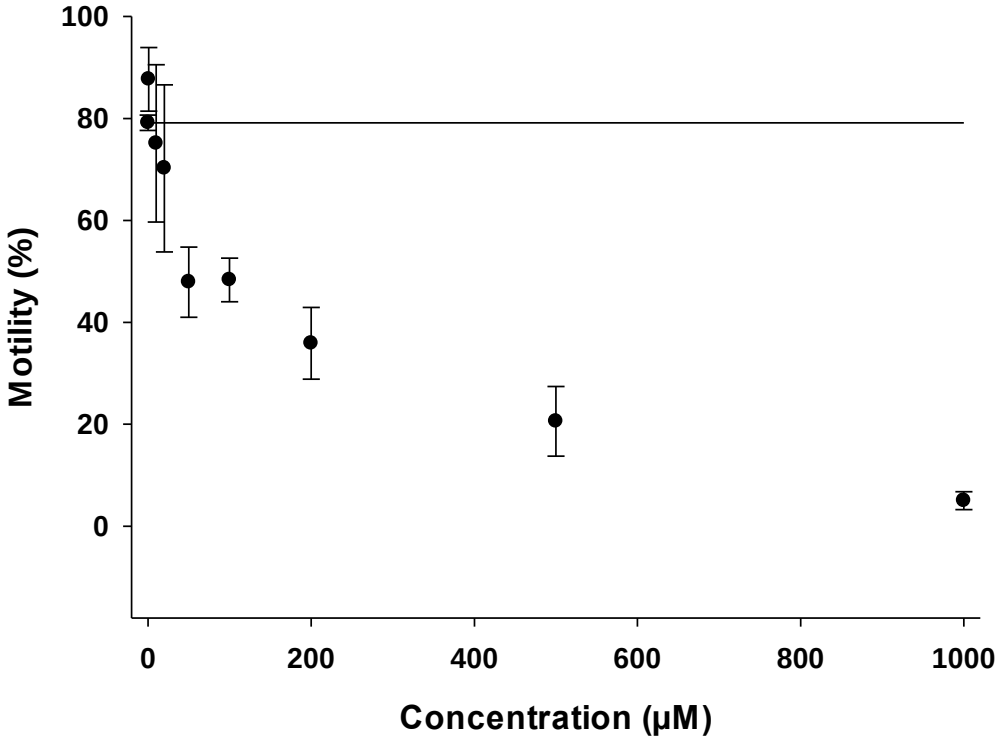


Figure 3.13. Sperm motility in the presence of the T-type voltage-gated calcium channel inhibitors, $NiCl_2$ and $ZnCl_2$. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The T-type voltage-gated calcium channel inhibitors $NiCl_2$ had a significant effect on motility starting at eight minutes and continued on through 20 minutes relative to the positive and negative controls (trypsin and CFR respectively). $ZnCl_2$ had little effect on motility and only after 16 minutes incubation was there a slight, but significant, decrease in motility relative to the positive and negative controls (trypsin and CFR respectively). The control, phosphate-free Ringer, $NiCl_2$, and $ZnCl_2$ T_{50} were 17.38 minutes, 21.21 minutes, 7.97 minutes, and 14.07 minutes respectively with no significant statistical differences. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ** 0.001 to 0.01 and ns ≥ 0.05. CFR: Calcium-free Ringer.

A


100
80
60
40
20
0
Motility (%)
0
200
400
600
800
1000
Concentration (μM)

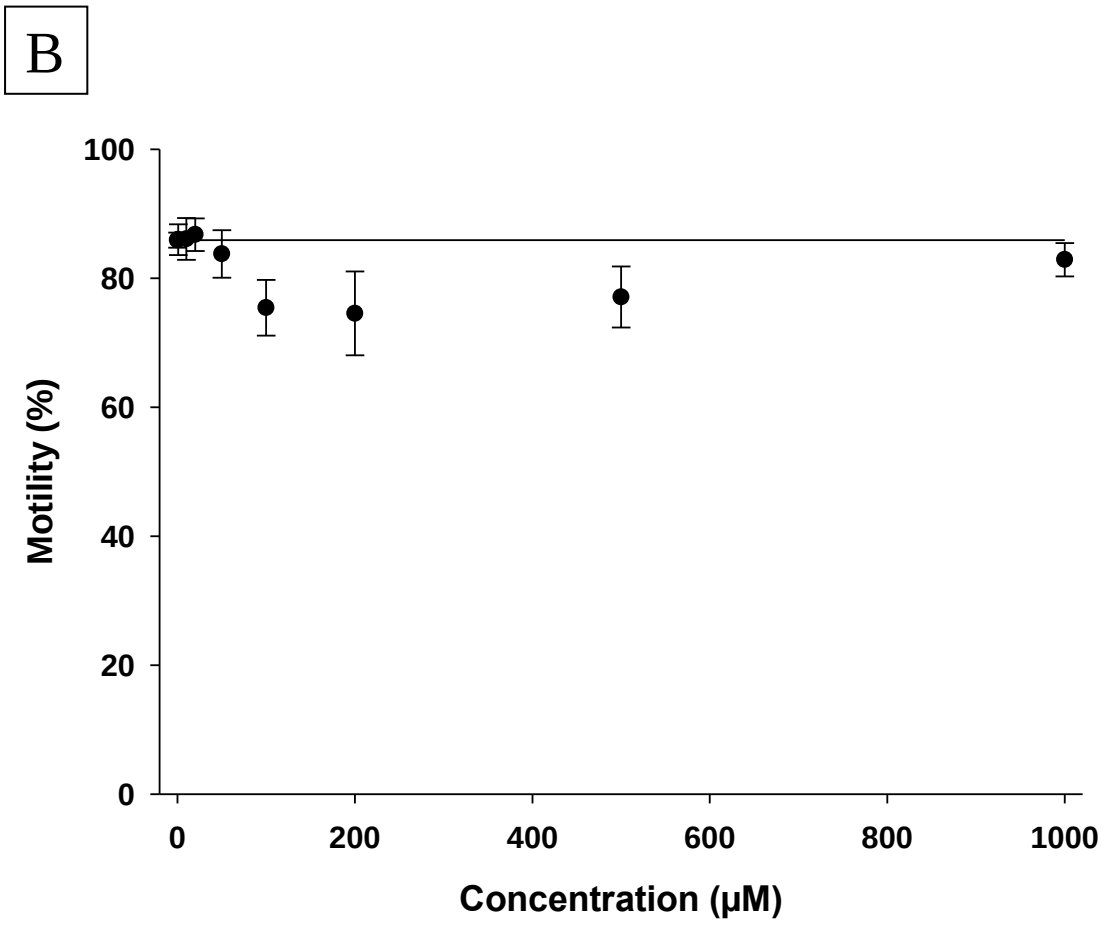


Figure 3.14. Sperm motility in the presence of varying concentrations of $NiCl_2$ and $ZnCl_2$ at 12 minutes. A) $NiCl_2$ and **B)** $ZnCl_2$. The percentage of sperm exhibiting flagellar beating was quantified at 12 minutes. Sperm were scored either as motile (flagellum beating) or immotile. $NiCl_2$ displayed a dose-dependent concentration curve with 50% motility inhibition continuing to occur at 50 µM relative to the control while $ZnCl_2$ had little effect on motility and only at 100 µM was there a slight, but significant, decrease in motility relative to the controls. Data shown are means ± SEM, with the control represented as a linear line, (n = 3 at each time point). Data is represented by averages and SEM bars, (n = 9 at each concentration). Using a t-test with equal variances not assumed p-value: **** < 0.0001, *** 0.0001 to 0.001, * 0.01 to 0.05, and ns ≥ 0.05.

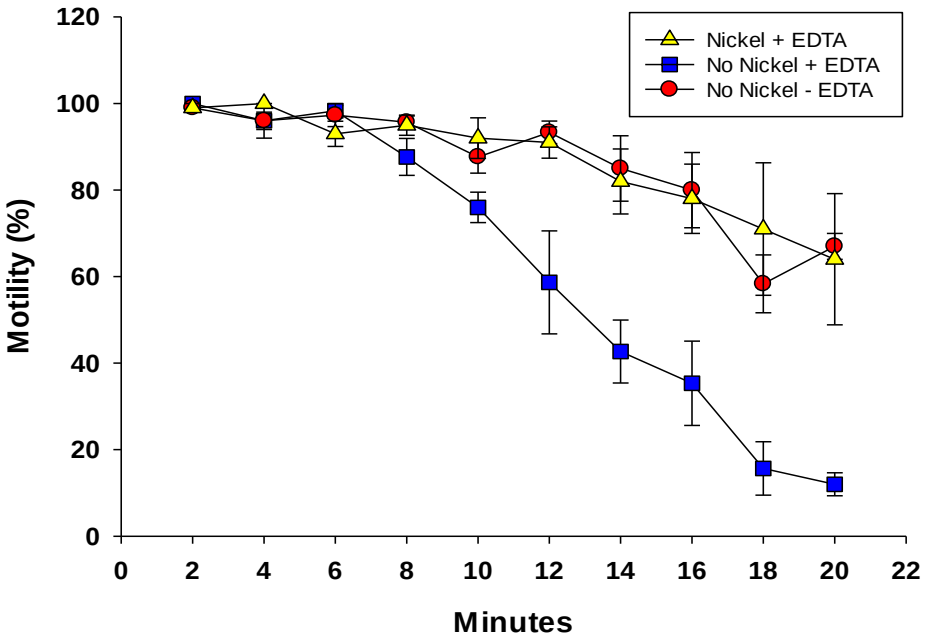


Figure 3.15. Sperm motility in the presence of 1 mM $NiCl_2$ and EDTA. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The control $NiCl_2$ + EDTA, no $NiCl_2$ + EDTA, and no $NiCl_2$ - EDTA T_{50} were 29.97 minutes, 13.22 minutes, and 28.84 respectively with a significant statistical difference only occurring between nickel + EDTA and no nickel + EDTA. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05.

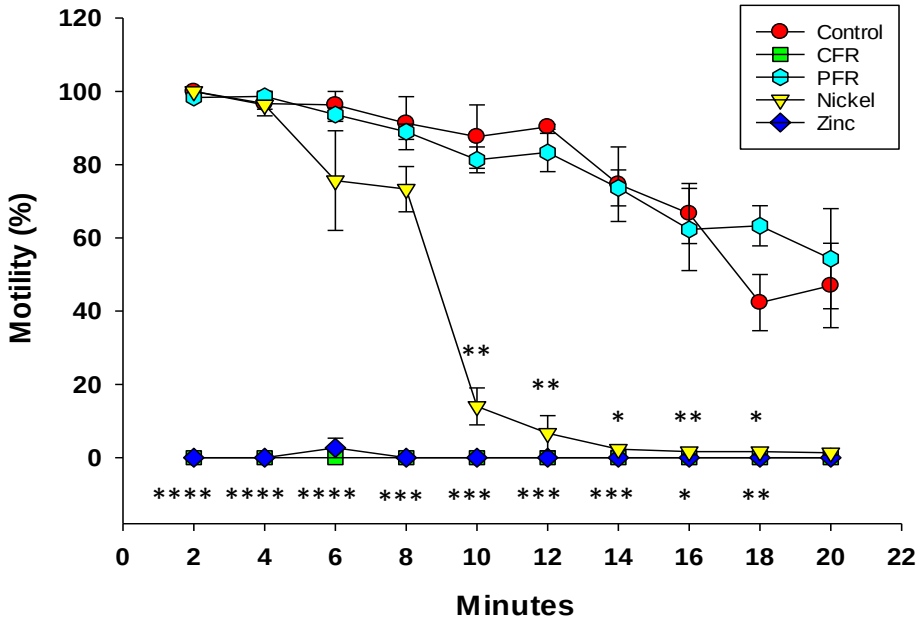
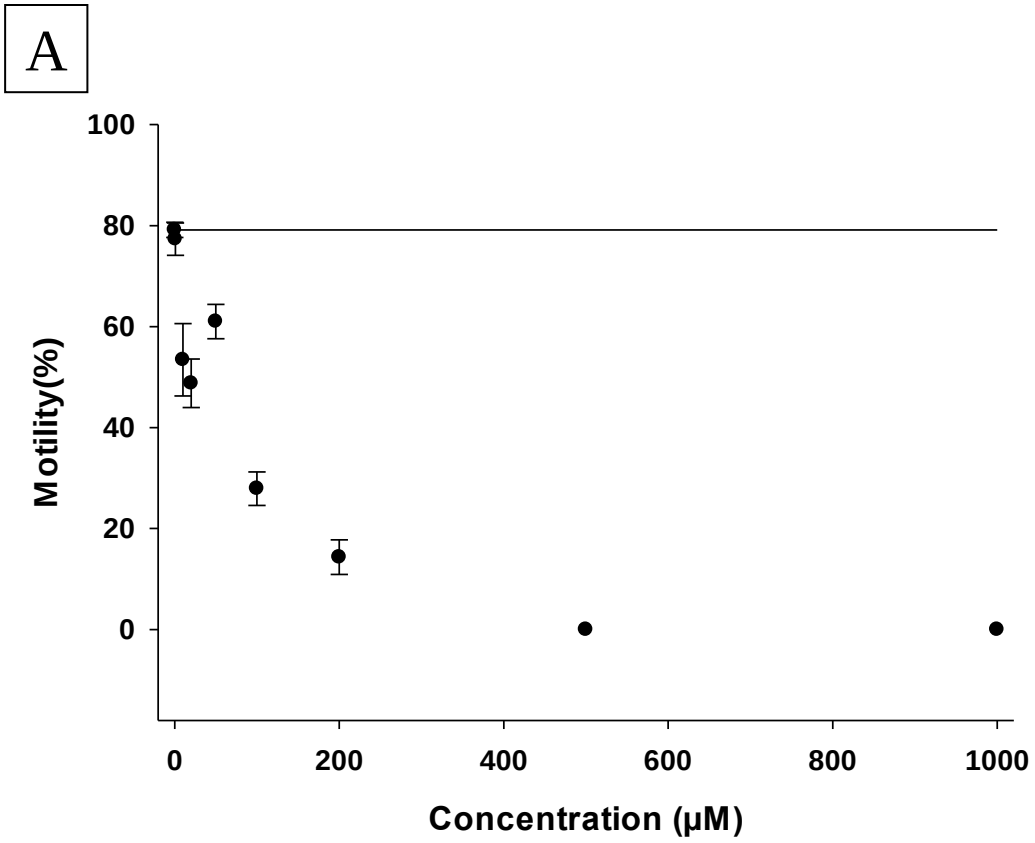


Figure 3.16. Sperm motility in the presence of T-type voltage-gated calcium channel inhibitors, $NiCl_2$ and $ZnCl_2$. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored as either possessing forward progressive motility or not. The T-type voltage-gated calcium channel inhibitor $NiCl_2$ displayed significant inhibition on forward progressive motility beginning at ten minutes relative to the positive and negative controls (control and CFR respectively) while $ZnCl_2$ exhibited a robust effect on forward progressive motility at the start of two minutes relative to the positive and negative controls (control and CFR respectively). The control, phosphate-free Ringer, $NiCl_2$, and $ZnCl_2$ T_{50} were 21.29 minutes, 22.63 minutes, 8.95 minutes, and 0 minutes respectively with significant statistical differences only occurring between phosphate-free Ringer and Zinc. Data shown are means ± SEM, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ** 0.001 to 0.01 and ns ≥ 0.05. CFR: Calcium-free Ringer.


A
100
80
60
40
20
0
Motility(%)
0
200
400
600
800
1000
Concentration (μM)

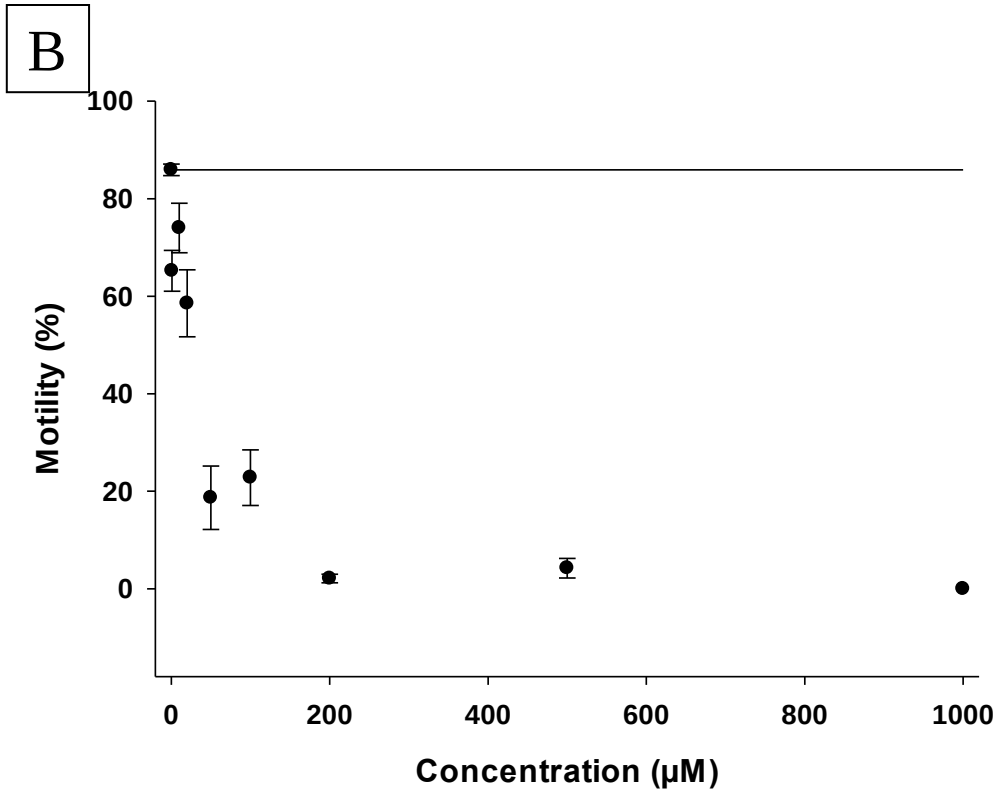


Figure 3.17. Sperm forward progressive motility in the presence of varying concentrations of $NiCl_2$ and $ZnCl_2$ at 12 minutes. A) $NiCl_2$ and **B)** $ZnCl_2$. The percentage of sperm exhibiting flagellar beating was quantified at 12 minutes. Sperm were scored either as either possessing forward progressive motility or not. $NiCl_2$ displayed a dose-dependent concentration curve with 50% motility inhibition continuing to as low as 10 µM relative to the control while $ZnCl_2$ also displayed a dose-dependent concentration curve and had a strong effect on forward progressive motility with 50% inhibition continuing to occur around 20 µM relative to the control. Data shown are means ± SEM, (*n* = 3 at each time point). Data is represented by averages and SEM bars, with the control represented as a linear line, (*n* = 9 at each concentration). Using a t-test with equal variances not assumed p-value: **** < 0.0001, ** 0.001 to 0.01, * 0.01 to 0.05, and ns ≥ 0.05.

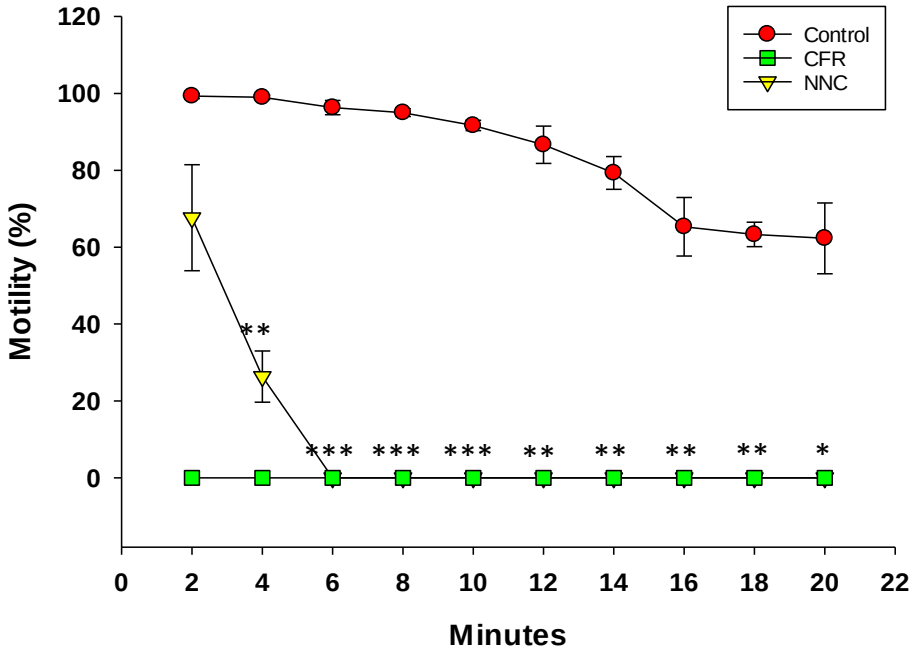


Figure 3.18. Sperm motility in the presence of highly selective T-type voltage-gated calcium channel blocker, NNC 55-0396 dihydrochloride. The percentage of sperm exhibiting flagellar beating was quantified at two-minute intervals for 20 minutes. Sperm were scored either as motile (flagellum beating) or immotile. The highly selective T-type voltage-gated calcium channel blocker, NNC 55-0396 displayed a very robust and significant effect on sperm motility beginning at four minutes, continuing for the remainder of the 20 minutes relative to the positive and negative controls (control and CFR respectively). The control and NNC 55-0396 T_{50} were 25.15 minutes and 3.79 minutes respectively with a significant statistical difference. Data shown are means ± SEM, (n = 3 at each time point). Data is represented by averages and SEM bars, (n = 3 at each time point). Using a t-test with equal variances not assumed p-value: ns ≥ 0.05. CFR: Calcium-free Ringer.

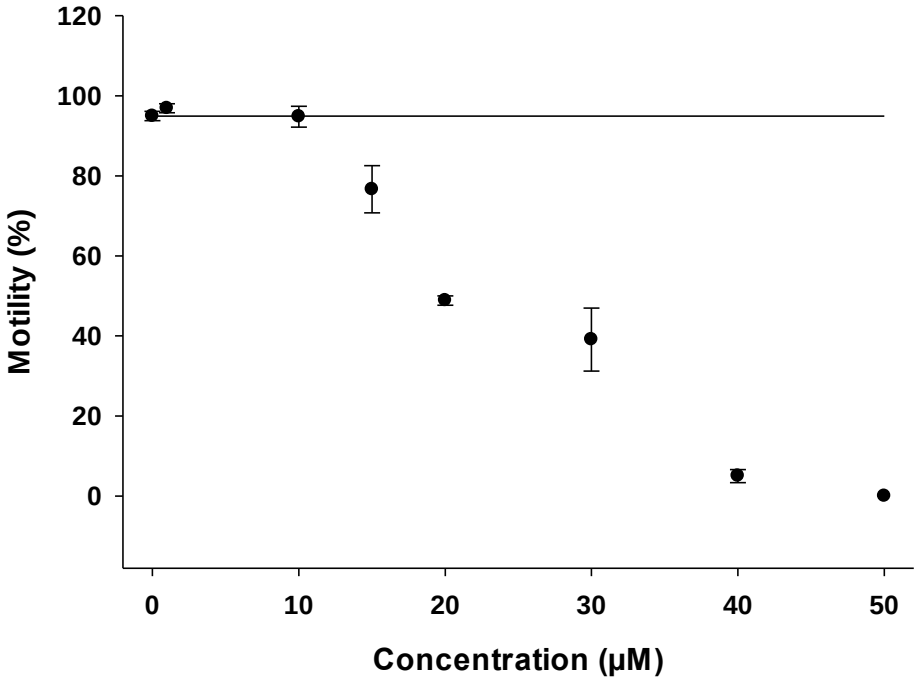


Figure 3.19. Sperm forward progressive motility in the presence of varying concentrations of highly selective T-type voltage-gated calcium channel, NNC 55-0396 at six minutes The percentage of sperm exhibiting flagellar beating was quantified at 12 minutes. Sperm were scored either as motile (flagellum beating) or immotile. NNC 55-0396 displayed a dose-dependent concentration curve with 50% motility inhibition continuing to as low as 20 µM relative to the control. Data is represented by averages and SEM bars, with the control represented as a linear line, (n = 9 at each concentration). Using a t-test with equal variances not assumed p-value: **** < 0.0001, *** 0.0001 to 0.001, * 0.01 to 0.05, and ns ≥ 0.05.

Agonist/ Antagonist	Minutes									
	2	**4**	**6**	**8**	**10**	**12**	**14**	**16**	**18**	**20**
Control	98.51	97.14	95.25	92.74	88.33	84.11	78.29	68.85	61.40	56.29
DIDS	100	100	97	96.66	84	82.66	75	71.66	59.66	57
VRPML	97	98.33	96	95.33	93.66	87.3	78.33	71.66	64.66	55.33
LCD	100	99.66	99	98.33	95	94.33	89.66	91	93.66	83
AMTD	94	87	82.33	77.66	68.66	58	56.66	51.66	46.33	40.66
MEM	96.33	95	92.66	83.66	76	57.66	50	51.33	35.66	22.66
IFEN	96	90.66	88.66	85	82.66	79	75	68	84.66	43.33
DAP5	99.66	97	93.66	92	86.66	83.33	77.66	67.33	66.33	41
cADPR	100	100	100	94.33	95.66	87.66	88.33	72.33	71	63.33
$NiCl_2$ (GM)	91	93.33	78.67	46.33	14.67	11.33	5.33	4	3.33	5.67
$NiCl_2$ (FPM)	96.33	96.33	75.66	73.33	14	6.66	2.33	1.66	1.66	1.33
$ZnCl_2$ (GM)	98.33	96.33	87.67	91	80	68	55.33	28.33	12	13.33
$ZnCl_2$ (FPM)	0	0	0	0	0	0	0	0	0	0
NNC	67.66	26.33	0	0	0	0	0	0	0	0

Table 3.4. Quantitative summary of inhibitors used over 20 minutes. DIDS: 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid, VRPML: verapamil, LCD: L-*cis*-diltiazem, AMTD: amantadine, MEM: memantine, IFEN: ifenprodil, DAP5: D-AP5, cADPR: 8-Br-cADPR, NNC: NNC 55-0396 dihydrochloride, GM: general motility, FPM: forward progressive motility.

Chapter 4: Conclusion

Insect sperm behavior and physiology is a highly understudied aspect of entomology. Many characteristics of insect sperm biology are unknown; therefore, this dissertation and series of experiments serves as a novel and significant contribution to insect physiology and mosquito biology.

Calcium is a necessary component in sperm motility and through series of experiments has shown that in the absence of calcium, the sperm are incapable of flagellar beating and forward progressive motility. *Culex pipiens* mosquito sperm also require activation through a type of serine protease located in the accessory glands of the male reproductive tract. Trypsin, a type of serine protease, has shown to be sufficient in activating sperm motility and has been labeled as an endogenous activator of sperm motility in *Culex* sperm. Increased phosphorylation also has been shown to play a role in sperm motility activation and is sufficient in the activation of sperm motility. Using okadaic acid, a phosphatase inhibitor, sperm in the presence of increased phosphorylation and the presence of calcium are able to bypass activation via trypsin. Sperm in the presence of okadaic acid and the absence of calcium lead to backwards motility, of which the mechanisms is still unknown. Backwards motility has been seen in only one other species, *Drosophila melanogaster*, which acts as means for sperm to navigate backwards into the female reproductive tract (Kottgen et al., 2011). While the mechanism for this backwards swimming behavior in *Culex* is still unknown, it creates important questions both in regard to its physiological importance in either the male or female reproductive tract *in vivo,* and the specific molecular targets within the axoneme that could result in this flagellar behavior. Whether this behavior is an evolutionarily conserved mechanism

amongst all Dipteran insects, or an alternative method of fertilization similar to that in *Drosophila melanogaster*, will require future studies. Future experiments should include calcium titrations along with influencing phosphorylation levels and observe how those aspects play a role in backwards motility.

An abundance of research has been executed analyzing the highly expressed protein and pH sensitive and voltage-gated channel CatSper that is a necessary component in calcium mobilization in many organism including mammals and some aquatic animals. Studies have shown that the manipulation of CatSper leads to male sterility. Unfortunately, following proteomic sequencing, Culex mosquito sperm do not possess nor express CatSper and therefore their calcium mobilization must be occurring through another protein. Following proteomic sequencing and protein compilation, a series of agonists and antagonists were used to individually test these series of proteins. The proteins tested included the voltage-dependent anion selective channel, an L-type voltage-gated calcium channel, the NMDA receptor, the ryanodine receptor, a cyclic nucleotide gated cation channel, and a T-type voltage-gated calcium channel. After examining the results of the specific antagonists on these proteins, it was determined that all but the T-type voltage-gated calcium channel inhibitors, $NiCl_2$ and $ZnCl_2$, had little to no effect on the mobilization of calcium and displayed no impact on the motility of the sperm.

$NiCl_2$ displayed a statistically significant effect on general sperm motility over the course of 20 minutes with a T_{50} of approximately eight minutes while $ZnCl_2$ did not displayed inhibition on general motility as the T_{50} was approximately 14 minutes. To

confirm that $NiCl_2$ was not exhibiting a lethal effect on the sperm, EDTA a chelator with a high affinity for metals including nickel was utilized. It was concluded that sperm in the presence of NiCl2, EDTA, and calcium, was in fact inhibiting sperm motility and did not possess any lethal effects. Further analysis was conducted using a dose dependent curve. $NiCl_2$ displayed a clear linear regression in sperm motility inhibition as the concentration increased while $ZiCl_2$ remained ineffective as concentrations increased. Forward progressive motility was then analyzed of which, $NiCl_2$ followed a similar trend as with general motility with a T_{50} of approximately nine minutes. $ZnCl_2$ however, displayed a significant effect on forward progressive motility over the course of 20 minutes with no sperm displaying forward progressive motility. Dose dependent curves were then created and analyzed, of which both $NiCl_2$ and $ZnCl_2$ displayed a linear regression in the inhibition of sperm motility as concentrations increased. Following these data, the robust effect of $NiCl_2$ and $ZnCl_2$ on general sperm motility and forward progressive motility suggests that the T-type voltage-gated calcium channel is responsible for facilitating the calcium necessary for sperm motility. Therefore, a highly selective T-type voltage-gated calcium channel inhibitor NNC 55-0396 was used. In the presence of this highly selective antagonist at low concentrations, sperm were 50% inhibited at approximately four minutes and completely inhibited at six minutes. Utilizing a dose dependent curve, sperm displayed inhibition at a linear regression as concentrations increased. This is further confirmation that *Culex* sperm are utilizing the T-type voltage-gated calcium channel to facilitate the calcium necessary for sperm motility.

The T-type voltage-gated calcium channels are important transducers of membrane potential changes into intracellular calcium signals that begin various physiological events. Calcium entering the cell through these voltage-gated calcium channels result in the initiation of many cellular pathways (Catterall 2011). The T-type voltage-gated calcium channel found in the *Culex* sperm activate at small and very negative depolarizations, inactivate quickly, and deactivate slowly while high-voltage activated channels require much larger and less negative membrane potentials (Catterall, 2011; Perez-Reyes, 2003). This allows calcium influxes through these low voltage T-type voltage-gated calcium channels to stimulate low-threshold spikes which lead to bursts of action potentials and recover quickly from inactivation which allows them to contribute to these bursts (Perez-Reyes, 2003). These characteristics allow sperm to activate rapidly and possess continuous motility in the presence of these constant calcium influxes. It also allows for swift directional changes from forward swimming sperm to backwards swimming sperm in the presence of increased phosphorylation.

Although incomplete, detailed studies regarding these various waveforms were investigated using SpermQ, a software that provides a quantification of flagellar beating using darkfield microscopy. Original darkfield videos possessing a rate of 30 frames/sec were increased to a rate of 100 frames/sec through the use of a high-powered Edgertronic (Edgertronic, SC1, Campbell, CA) camera. This allowed waveform generation to be reanalyzed using darkfield videos taken at a higher frame rate. This information provided allows us to qualify and quantify the relationship between waveforms and the effect of inhibited calcium on waveforms.

As discussed in chapter two, it has been previously reported that *Culex* mosquito sperm possess three distinct waveforms, A, B, and C (Thaler et al., 2013). However, newly acquired data using a high-powered camera (Edgertronic camera; Edgertronic, SC1, Campbell, CA) suggests that *Culex* sperm may possess only two distinct flagellar waveforms, a non-hyperactivated and hyperactivated form. The low frame rate acquisition of the camera used at the time may have resulted in waveform B as an artifact and just a transition waveform between the non-hyperactivated and hyperactivated states. In mammals, ejaculated sperm initially display a symmetrical waveform but following capacitation, undergo an asymmetrical waveform and hyperactivated motility (Suarez 2008). As demonstrated by SpermQ, at reduced velocities, sperm flagellar beating is highly symmetrical while sperm at increasing velocities possess erratic and asymmetrical flagellar beating. Variability occurs between the lower and higher velocities suggesting that the sperm possess transitional waveforms between states of symmetrical and asymmetrical flagellar beating.

In the presence of calcium inhibitors $NiCl_2$ and $ZnCl_2$ and an environment in which extracellular calcium is reduced by 50%, the sperm still possess a strong beat frequency relative to the control sperm. On average, the amplitude of their flagellum was only slightly dampened however, a symmetrical flagellar wavelength was maintained. Although sperm in the presence $ZnCl_2$ still maintain a symmetrical waveform, most of the sperm do not possess forward progressive motility and in the case of sperm in the presence of $NiCl_2$, sperm do not exceed a velocity of approximately 40 µm/sec. This reduction in calcium in the presence of either $NiCl_2$ or $ZnCl_2$ may prevent dynein motors

from shifting significantly. This reduction in microtubule shifting may result in a lack of a bend in the flagellum that applies enough force to drive a high amplitude and forward progressive motility.

To determine if reduced calcium leads to a dampened amplitude and inhibited forward progressive motility, calcium levels would need to be titrated and the quantification of amplitude and velocity measured and compared through the use of SpermQ. Expanding on this data to further and more closely study the flagellar behavior would significantly advance the field of insect physiology and insect sperm behavior.

Furthermore, to expand our knowledge of the effects of the T-type voltage-gated calcium channel plays in *Culex* sperm motility, genetic modifications and targeted mutagenesis could be applied. Genome editing techniques include transcription activator-like effector nuclease (TALENs), zinc finger nuclease, or through the CRISPR-Cas9 system. Either genetic silencing or genetic knockout of the gene(s) responsible for the protein that regulates the calcium necessary for sperm motility may confirm and support the necessity of the T-type voltage-gated calcium channel in sperm locomotion. Following the successful targeted mutagenesis could result in genetically modified mosquito lines containing males incapable of fertilizing eggs, leading to a potential insect control method.

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Appendix: Waveform Generation Analysis

Abstract

In the presence of endogenous activator, trypsin, a serine protease, and calcium, the *Culex* mosquito sperm are capable of possessing three distinct waveforms. As these wavelengths progress, so do their velocities and progression forward. Detailed studies regarding these various waveforms were investigated using SpermQ, a software that provides a quantification of flagellar beating using darkfield microscopy. Original darkfield videos possessing a rate of 30 frames/sec were increased to a rate of 100 frames/sec through the use of a high-powered Edgertronic camera. This allowed waveform generation to be reanalyzed using darkfield videos taken at a higher frame rate. Sperm in the absence of calcium were also analyzed with SpermQ for the first time. As calcium was inhibited and unavailable to the sperm, the waveforms, velocities, and forward progressive movement shifted, thereby altering beat frequencies and movement of the sperm through space. This information provided allows us to qualify and quantify the relationship between waveforms and the effect of inhibited calcium on waveforms.

Introduction

Culex sperm are initially quiescent and are activated by male reproductive secretions from the accessory glands that contain a serine protease (Thaler et al., 2013; Stephens et al., 2017). Once motile, these sperm require an increase in intracellular calcium to maintain flagellar beating resulting in forward progressive motility. Together, these two components initiate a series of downstream phosphorylation events through a MAP kinase (MAPK) pathway that presumably phosphorylates some target within the axoneme.

The eukaryotic sperm flagellum is a complex structure consisting of hundreds of proteins used to generate and regulate motility The underlying microtubule-based structure, the axoneme, is evolutionarily conserved across taxa with modifications in some animal systems, including insects (Gibbons 1981; Satir and Avolio 1986; Inaba, 2011). The stereotypical axoneme, found in most animal cilia and flagella, consists of nine outer microtubule doublets surrounding a central pair of microtubules culminating in a canonical "9+2" structure. In contrast, most insect sperm flagella possess an additional ring of accessory microtubules resulting in a modified "9+9+2" axoneme. Unlike the inner ring that consists of nine double microtubules and a pair of dynein motors on each doublet, the outer ring of microtubules in many insect taxa is made up single microtubules consisting of 15-16 protofilaments each (Dallai et al., 1993). Mosquitoes possess this outer ring but, in addition, vary from the canonical axonemal structure by possessing only a single microtubule in the center instead of a central pair, yielding a "9+9+1" axoneme (Dallai et al., 1993). At the distal portion of the flagellum, the

axoneme no longer contains either the outer ring of microtubules or the central rod; in addition, the doublet microtubules are reduced to singlets (Justine et al., 1988). This "9+9+1" structure is believed to be a synapomorphy (a shared and derived character common between an ancestor and its descendants) of mosquitoes (Justine et al., 1988; Dallai et al., 1993).

The ability of the axoneme to bend creating flagellar movement is due to the sliding of axonemal doublet microtubules through the action of axonemal dynein located on the inner and outer arms of microtubule doublets (Gibbons 1981; Satir and Avolio 1986; Inaba, 2011). Dyneins are molecular motors that hydrolyze ATP hydrolysis which, as a result, generates a force called a "power stroke" resulting in the bending of the sperm flagellum (Burgess et al., 2003). Dyneins are minus end directed motors that push the microtubule doublets towards the flagellar base. Microtubules, including those in axonemes, form from the "-"end to the "+" end and in the case of axonemes in cilia and flagella the "- end" is found at the base of the flagellum whereas the "+ end" is at the distal end of the (Lindemann and Lesich, 2020). In cells in which cytoplasmic dynein carries a range of cargo, that cargo moves from the "+ end" of the microtubules to the "- end_. In the case of the axoneme, each microtubule doublet serves as the cargo that is carried to the base of flagella when the motors are active (Lindemann and Lesich, 2020). The stalk of each dynein heavy chain has a binding domain for the microtubules to attach to the adjacent doublet (Lindemann ad Lesich, 2020).

In sperm from many animal taxa, the energy for microtubule sliding in the axoneme is provided by ATP that is produced in the mitochondria or through the action

of glycolytic enzymes (Werner et al., 1999). In most species of flightless insects, the mitochondria go through a metamorphosis during spermiogenesis in which the mitochondrial structure is structurally modified (Bao et al., 1992). The mitochondria in early spermatids fuse near the nucleus and form a round body where they are later separated into two bodies that fuse into large mitochondrial derivatives (Werner et al., 1999). The mitochondrial derivatives in insect sperm comprise a paracrystalline structure embedded in a matrix and extend alongside the axoneme making up a large percent of the cell's volume (Bao et al., 1992). This paracrystalline array may result from the reorganization of cristae proteins in the mitochondria (Bao et al., 1992). Although most animal sperm use oxidative phosphorylation for axonemal movement and sperm energy, in insect sperm, the paracrystalline matrix does not appear to possess either cytochrome oxidase or succinic dehydrogenase activities that are necessary for cellular respiration (Bao et al., 1992). In the mosquito *Culex quinquefasciatus*, cytochrome c oxidase activity was mostly observed in the mitochondrial cristae of early spermatids but not at high levels in mature sperm (Bao et al., 1992). These results were similar to *Drosophila melanogaster* sperm which confirmed the activity of cytochrome c oxidase but only at weak and barely visible levels (Perotti et al., 1973). However, the metabolic pathways that are responsible for insect sperm motility currently remain unknown.

Thaler et al., (2013) observed three distinct flagellar waveforms possessed by *Culex* sperm in the presence of the endogenous activator, trypsin, and calcium. These waveforms consisted of waveform A, a low-amplitude, long-wavelength waveform, waveform B, a low-amplitude, short-wavelength superimposed on a high-amplitude,

long-wavelength waveform, and waveform C, a double waveform converted into a single helical wave (Thaler et al., 2013). As these wavelengths progress from A to C, so did their velocity and progression forward (Thaler et al., 2013). This is in contrast to other organisms sperm including mammalian sperm that do not possess distinct waveforms but instead, the sperm display hyperactivation or lack thereof. This hyperactivated state in mammalian sperm involves transition from a symmetrical, low amplitude flagellar beating to asymmetrical, high amplitude beating (Zaferani et al., 2021).

As *Culex* sperm flagellar behavior has yet to be quantified, SpermQ software was utilized to allow further analysis of flagellar performance including a look into beat frequency, velocity, flagellar amplitude, and average curvature of sperm in various conditions.

Materials and Methods

Mosquitoes and Dissections

The *Culex pipiens* wild type line were maintained at 27°C, 80% relative humidity, 12:12 light/dark cycle, and given sugar cubes as a sugar source. The *C. pipiens* line is autogenous, meaning a blood meal is not required for the females to lay egg rafts, therefore only sugar was provided for the females. Egg rafts were collected in trays of recycled larval water and were hatched in cups containing deionized water where they were then placed into larger pans. The larvae fed on a 2:1 diet of mouse food and brewer's yeast with 1.8 g fish oil per 25 g mouse food and brewer's yeast. Pupae were collected using a sieve and added into cups with the remainder of the larvae water. Cups

were added into mosquito cages and allowed to eclose for four days. On the fourth day, the pupae cups were strained into oviposition trays which remained in the mosquito cages for two days to allow females to lay eggs. The adult mosquitoes were collected using a battery powered aspirator and were anesthetized and sorted using CO_2. Males were then placed individually in 1.25-ounce Solo cups (product #P125, Solo Cup Company) with lids (product #PL1, Solo Cup Company, Lake Forest, IL). Chloroform-soaked cotton balls were introduced to the cups as a method of sacrificing the mosquitoes. The male reproductive tract was then dissected out in either phosphate buffered saline (PBS): 20 mM Na_2HPO_4, 100 mM Na_2HPO_4 or the Insect Ringer of interest (recipes below) and placed on a clean glass side with the appropriate Insect Ringer. All sperm used in these experiments were mature sperm located in the seminal vesicles. Unless otherwise stated, testes and accessory glands were removed prior to the assay leaving only the seminal vesicles.

To test general sperm motility under various conditions, the sperm were placed in a total of 20µl of varying Insect Ringers. Insect Ringers that were used for experiments: The seminal vesicles were squeezed open using forceps in Insect Ringer to release the sperm. A coverslip with clay at each corner was added to the top of the slide and pressed down until the coverslip touched the liquid. This allowed the sperm to move freely in three dimensions. The slide was then moved to a microscope stage and the behavior was monitored on a darkfield microscope (Nikon Labophot, Melville, NY) with a high-speed camera (Edgertronic, SC1, Campbell, CA).

Sperm that were analyzed using SpermQ included sperm incubated in one of the following series of dilutions: Insect Ringer containing trypsin and calcium, Insect Ringer containing trypsin, calcium, and 1 mM $NiCl_2$, or PFR containing trypsin, calcium, and 1 mM $ZnCl_2$. During the 10 second recordings, the microscope stage was shifted three times to account for overall sperm behavior in the solution. These behavioral recordings were then analyzed using image processing software (ImageJ, NIH). This was repeated for each biological replicate.

Insect Sperm Media

Insect Ringer: 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 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, Insect Ringer without phosphates (PFR): 110 mM NaCl, 5 mM KCL, 0.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $NaHCO_3$, 1 mM glucose, 20 mM HEPES, pH 7.2.

Darkfield and High Frame Rate Imaging

An upright Nikon microscope (Melville, NY) equipped with a high-speed camera (Edgertronic, SC1, Campbell, CA) was used to record sperm motility videos in darkfield. Images were taken using a 20X objective lens at 100 frames/sec for 10 seconds resulting in a compilation of 1,000 images.

Image Processing and Analysis

Darkfield images were uploaded into imaging software ImageJ (NIH) which was used to calibrate the digital pixel distance value into the appropriate microns. These images were saved as image sequences and after selecting frames of interest containing a sperm of interest were imported back into ImageJ. The imported images were then converted into a stacked TIFF file. These processed images were further analyzed using SpermQ, image processing software package that quantifies sperm motility. Following loading the stacked images into SpermQ, a region of interest is selected which contains an image region where the sperm is present over the entire sequence (greater than or equal to one second) (Hansen et al., 2018). Setting this region of interest decreases the regions where SpermQ is searching for the flagellum, increasing the accuracy of the software (Hansen et al., 2018). SpermQ traces the sperm head by utilizing the brightest intensity into points then traces the flagellum with normal lines and a Gaussian curve (Figure A.1). In order to visualize these results, data was uploaded into SpermQ Evaluator which creates overview tables, data sets, and visual representations of both a single flagellar beat cycle and the movement of the head through space.

Results

Beat Frequency and Velocity of Sperm

SpermQ was used to quantify flagellar beat frequency and sperm velocity in the presence of calcium (Figure A.2). SpermQ provided a visual map of a single flagellar beat cycle and the period for that beat cycle. SpermQ also provided the movement of the

head of the sperm through space. When sperm were in the presence of only Insect Ringer and trypsin, they displayed reduced beat frequencies and velocities and displayed symmetrical flagellar beating similar to the symmetrical beating in non-hyperactivated mammalian sperm. As flagellar waveforms became more asymmetric, both beat frequency and velocity increased, comparable to the flagellar behavior of mammalian sperm in a hyperactivated state.

Analysis of Beat Frequency and Velocity

SpermQ was utilized to quantify mosquito sperm beat frequency and velocity under various environmental conditions including in the presence and absence of calcium. In the presence calcium, there was a positive linear correlation between sperm velocity and beat frequency (Figure A.3). When calcium entry through the presumptive T-channel (Chapter 3) was inhibited by, $NiCl_2$ or $ZnCl_2$, beat frequency was variable but was indistinguishable from controls. In contrast, sperm velocity was detectable reduced and did not exceed 40 μm/sec (Figure A.3) in the presence of these inhibitors

Flagellar Amplitude

Flagellar beat amplitudes were also analyzed, as the sperm moves through space. Sperm in the presence of $NiCl_2$ and $ZnCl_2$ displayed a greatly reduced velocity but, interestingly, maintain a beat frequency indistinguishable from controls. Further, under

conditions when calcium was inhibited by 50%, the sperm only possessed a slightly dampened amplitude relative to the control. These data imply that while T-channel calcium blockers inhibit progressive motility they do not affect either beat frequency of flagellar (Figure A.4).

Flagellar Curvature

Quantifying the average curvature of the flagellum over its length yielded information on the symmetry of the flagellar beat over time. Sperm that were immotile lacking any flagellar beating resulted in zero curvature while a positive or negative value distinguished the direction of the flagellar bend. In both the control and under conditions where calcium entry through presumptive T-channels was blocked by $NiCl_2$ or $ZnCl_2$, the flagellar beat was indistinguishably symmetrical (Figure A.5).

Discussion

It has been previously reported that *Culex* mosquito sperm possess three distinct waveforms. This newly collected data suggests that *Culex* sperm may possess only two distinct flagellar waveforms. In mammals, ejaculated sperm initially display a symmetrical waveform but following capacitation, undergo an asymmetrical waveform and hyperactivated motility (refs). As demonstrated by SpermQ, at reduced velocities, sperm flagellar beating is highly symmetrical while sperm at increasing velocities possess

erratic and asymmetrical flagellar beating. Variability occurs between the lower and higher velocities suggesting that the sperm possess transitional waveforms between states of symmetrical and asymmetrical flagellar beating.

In the presence of calcium inhibitors $NiCl_2$ and $ZnCl_2$ and an environment in which extracellular calcium is reduced by 50%, the sperm still possess a strong beat frequency relative to the control sperm. On average, the amplitude of their flagellum was only slightly dampened however, a symmetrical flagellar wavelength was maintained. Although sperm in the presence $ZnCl_2$ still maintain a symmetrical waveform, most of the sperm do not possess forward progressive motility and in the case of sperm in the presence of $NiCl_2$, sperm do not exceed a velocity of approximately 40 µm/sec. This reduction in calcium in the presence of either $NiCl_2$ or $ZnCl_2$ may prevent dynein motors from shifting significantly. This reduction in microtubule shifting may result in a lack of a bend in the flagellum that applies enough force to drive a high amplitude and forward progressive motility.

In order to determine if reduced calcium leads to a dampened amplitude and inhibited forward progressive motility, calcium levels would need to be titrated and the quantification of amplitude and velocity measured and compared through the use of SpermQ.

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Figures and Tables

Figure A.1. Tracking mosquito head movement using SpermQ.

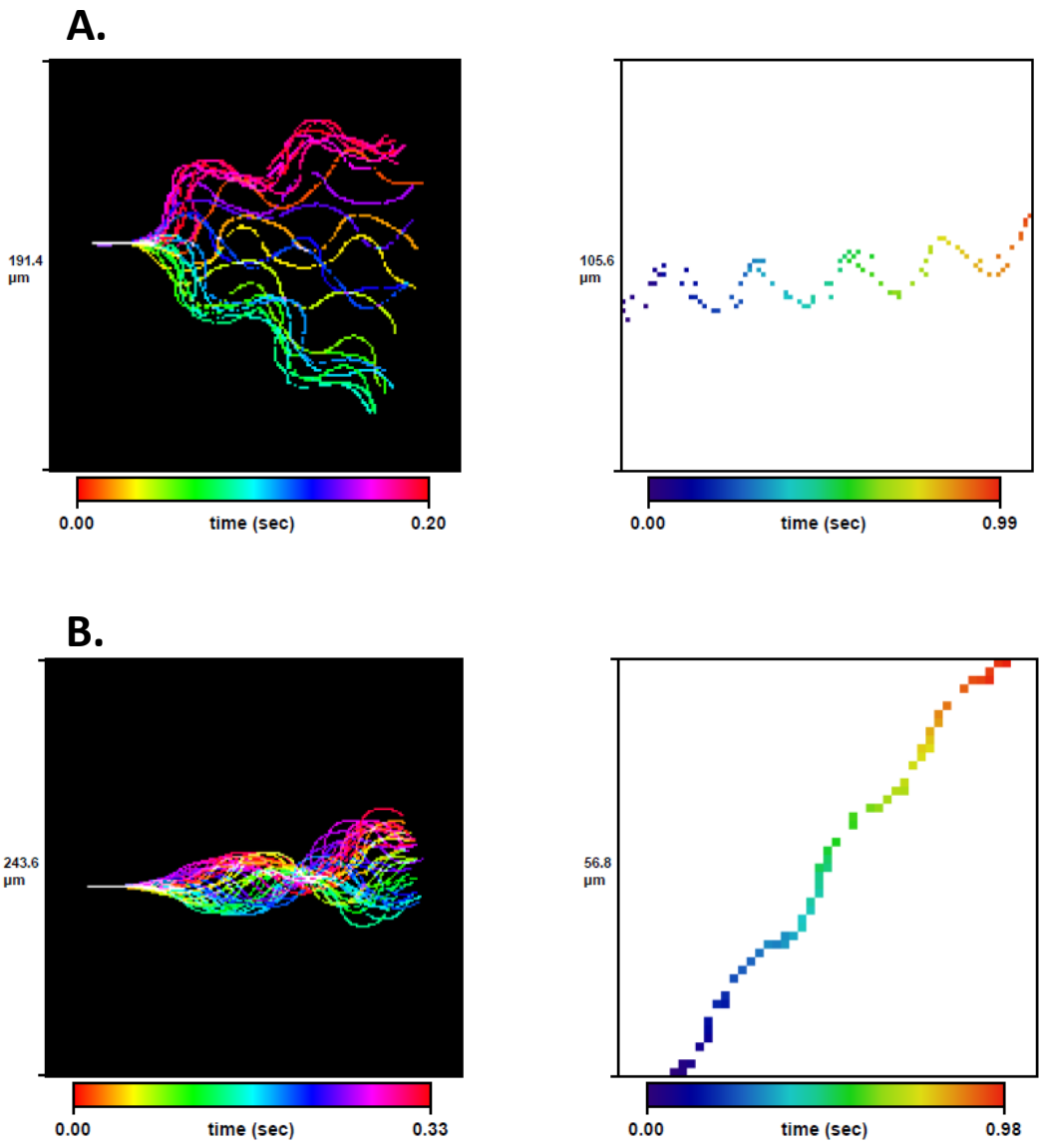

Figure A.2. Sperm beat frequencies and movement through space. A) Sperm with a beat frequency of 5.05 and a velocity of 110.29 possess a much more erratic and asymmetrical flagellar beating compared to **B)** sperm with a beat frequency of 3.00 and a velocity of 74.74 µm/sec which has a symmetrical flagellar beat.

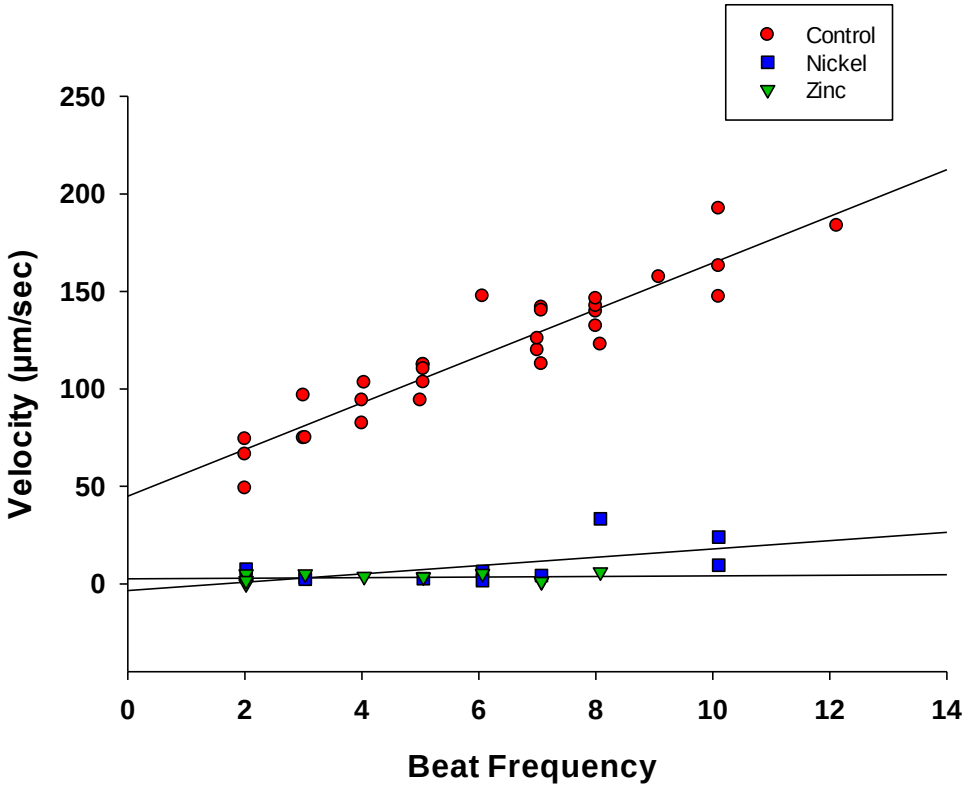

Figure A.3. Beat frequencies and velocities of sperm in the presence and absence of calcium. Sperm in the presence of calcium (control) possess increasing velocities with growing beat frequencies and transition from a non-hyperactivated to a hyperactivated state which include many transitional states. Sperm in the absence of 50% of extracellular calcium (nickel and zinc) still maintain beat frequencies comparable to the controls but maintain a low to nonexistent velocity and never exceed 40 µm/sec. (n = 30 for controls and 10 for each nickel and zinc).

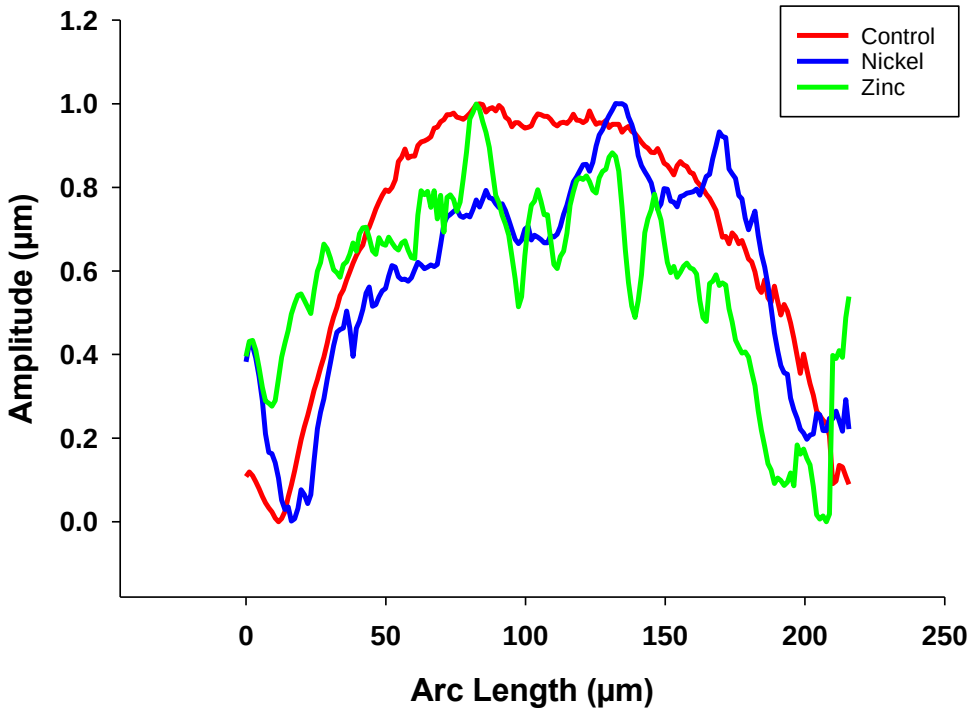

Figure A.4. Flagellar amplitude in the presence and absence of calcium. Data comparing sperm flagellar amplitudes. Amplitudes are slightly dampened in the absence of 50% extracellular calcium but remain similar to the control flagellar amplitudes. Data is represented as averages. (n = 30 for controls and 10 for each nickel and zinc).

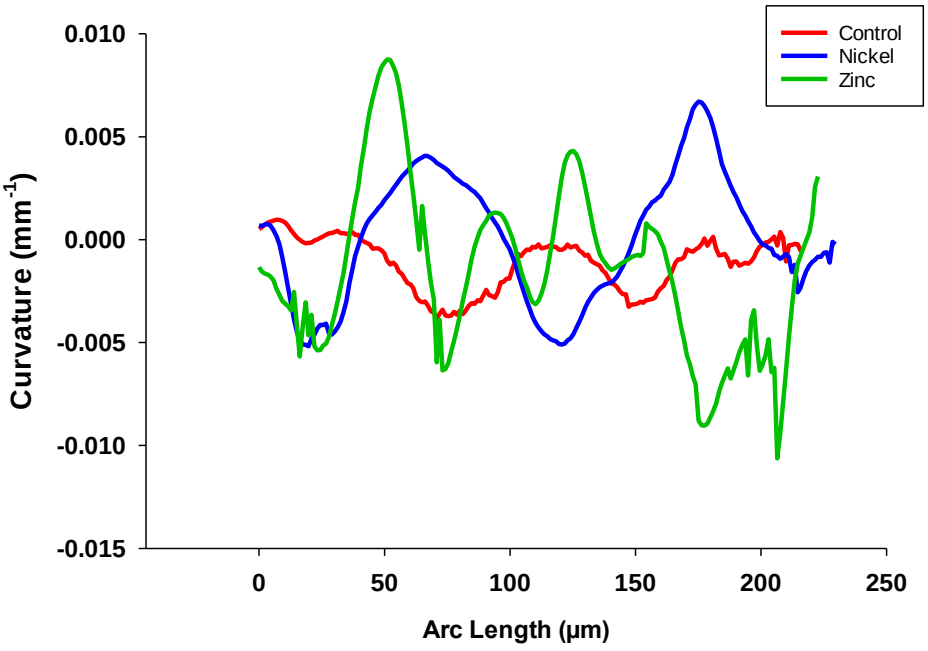

Figure A.5. Average flagellar curvature in the presence and absence of calcium. The average curvature for sperm in both the presence of calcium and sperm in the absence of 50% extracellular calcium display symmetrical beat frequencies across the length of the flagellum. Data s represented as averages (*n* = 30 for controls and 10 for each nickel and zinc).