ANALYSIS OF MOSQUITO SPERM FLAGELLAR WAVEFORM TO EVALUATE SIGNALING PATHWAYS NECESSARY FOR SUCCESSFUL FERTILIZATION

By

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ABSTRACT

Mosquitoes are vectors of many diseases that result in numerous human deaths worldwide. The overall goal of this study was to determine whether voltage-gated Calcium ion channels are required for the entry of Ca^{2+} into sperm to promote sperm motility. Previous studies on *Culex quinquefasciatus* showed that following activation, sperm progress through three distinct flagellar waveform patterns resulting in increased velocity and progressive motility. Additionally, it was found that in the absence of Ca^{2+} sperm remained immotile. When accessory glands/seminal vesicles are activated with trypsin and Ringer solution containing Ca^{2+} the sperm is able to progress through all three waveforms. When EGTA/okadaic acid substances were added and calcium was removed from the extracellular environment using calcium chelator, the sperm began to move backward. In these experiments, we investigated how different divalent cations affected mosquito sperm motility. Zinc and Nickel are used as they are known to be potential blockers to voltage gated Ca^{2+} ion channels. Therefore they are used as potential inhibitors of Ca^{2+} entry that would ultimately affect the downstream phosphorylation of flagellar proteins. Dark field microscopy and image processing were used to assess various flagellar parameters (e.g., flagellar beat frequency, mean head velocity, etc.) in response to the addition of Ni^{2+} and $Zn^{2+.}$ In the case of both cations, we observed significant decreases in those parameters relative controls. Particularly Zinc has shown strong correlation with decreased sperm motility. This indicates that voltage gated Ca^{2+} channels are necessary for entry of Ca^{2+} into sperm. This entry of Ca^{2+} influences sperm motility. Zinc can be used as a tool to help identify potential targets like the Ca^{2+} voltage gated ion channels that could be used to control mosquito populations.

ACKNOWLEDGEMENTS

I would like to express greatest gratitude towards my faculty mentor and professor, Dr. Richard Cardullo, for his patience and guidance throughout my undergraduate career at University of California, Riverside. He has been supportive with development and capstone research projects and my academic endeavors. I also want to give thanks to Dr. Catherine Thaler who taught me how to use Dark Field Microscopy and Image Processing software and gave me valuable advice while writing this paper. Lastly, I would like to thank Gabby Martinez and Kaira Carstens, my fellow laboratory members for guiding me through Edgertronic software and camera usage.

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Introduction:

I.Relevance - Mosquitoes are vectors to disease

Many of the mosquito vectored diseases including Malaria, Zika virus and Yellow fever are vector borne diseases transmitted by mosquitoes. Mosquitos cause approximately 230 million cases of malaria annually, killing over 400,000 people each year (World Health Organization, 2014). Even with this alarming high mortality rate, many mosquito borne pathogens do not have a commercially licensed vaccine. Further, diseases such as malaria that do have a short-term cure often encounter problems with drug resistance. Previously, mosquito control studies have focused on killing adults or larvae, instead of targeting the reproduction of mosquitoes themselves. Even when reproduction has been considered, more data has been collected on the female reproduction in mosquitoes and only limited studies have focused on males (Degner et al., 2016). Therefore, a promising alternative is to control mosquito vectored diseases by controlling mosquito populations through fertility regulation. The research in this capstone project involved targeting sperm motility by disrupting Ca^{2+} regulated channels with the aim of identifying potential inhibitors to Ca^{2+} channels that could block reproduction.

II. Basic Sperm Structure and Flagellar Motion

In mosquitos, sperm are initially quiescent during storage in seminal vesicles. A trypsin-like protease, an enzyme that breaks down proteins, activates protease receptors in the male accessory glands that lead to the activation of sperm motility (Haruhiko, 2011). The sperm activation event also requires an influx of extracellular Ca^{2+}, presumably through a plasma membrane-associated ion channel (Thaler et al., 2013). This Ca^{2+} influx triggers a mitogen

activated protein kinase (MAPK) mediated pathway that results in three different successive waveforms identified as waveforms A, B, and C (Figure 1) characterized by increased flagellar beat frequency, flagellar amplitude, and progressive velocity (Thaler et al., 2013) .

The mechanics of flagellar beating are well known. Axonemal motor proteins, specifically dynein, cause microtubule filaments to slide against each other generating the force that drives the flagellar beat. When dynein is bound to microtubules and undergoes ATP hydrolysis binding and conformational change occurs (Ishibashi et al., 2020).

Many eukaryotic sperm possess a "9+2" microtubule structure in their axoneome. The 9 +2 arrangement refers to how microtubules are organized in the flagellum. Nine pairs of microtubules situated on the outside that are bound together and two pairs of microtubules in the middle that are not bound together. Attached to the doublet pairs, are two dynein molecules that catalyze ATP hydrolysis allowing movement along microtubule (Bhabha et al., 2015). The axoneme of the insect sperm tail is usually described with the 9+9+2 designation which refers to 9 accessory tubules surrounding the classical 9 + 2 structure (Afzelius et al., 1993). (Figure 2).

III. Environmental factors that affect flagellar motion

Other than Ca^{2+} playing a role in affecting the direction of movement of the sperm, sperm motility is affected by other environmental stimuli as the flagellum is a sensor for other elements in the environment. Ni^{2+} (Yokoi et al., 2003) and Zn^{2+} concentration are known to influence sperm movement, waveform generation and flagellar frequency (Zhao et al., 2016). In the protist, Euglena, magnesium abolished the change in flagellar frequency. Meaning that there appeared to be no flagellar activity when external Mg2+ was placed in Calcium Free EGTA fluid of the ringer solution. The Ca^{2+} caused an increase in flagellar waveform reversal and a decrease

in beat frequency (Nicholes et al., 1980). In the southern house mosquito, *Culex quinquefasciatus*, the presence of Ca^{2+} determined the direction of sperm motility . With the lack of trypsin/accessory glands and the Ca^{2+} chelating agent, EGTA, and by promoting phosphorylation with okadaic acid, 100% of the sperm swam backwards. In the presence of trypsin protease and CFE ringer sperm remained immotile. The CFE ringer contained no Ca^{2+}. The sperm remaining immotile suggests that trypsin needs external Ca^{2+} to make the sperm motile. External Ca^{2+} influences the activation of forward progressive motility and continued motility. The Ca^{2+} also impacts the direction in which the sperm moves (Thaler et al., 2013).

IV. Analyzing the effects of Ni^{2+} and Zn^{2+} impact on sperm motility

In this study, I designed experiments to test whether specific divalent ions had an effect on sperm movement and motility. Specifically, I investigated if zinc and nickel ions can be used as potential inhibitors of Ca^{2+} entry affecting the downstream phosphorylation of flagellar proteins. Sperm flagellar waveforms and velocity were used to evaluate whether Ni and Zn had an effect on motility. If they did, it implies that Ca^{2+} gains entry into the sperm through a voltage gated Ca^{2+} channel. Sperm Q, a program that was developed to analyze flagellar patterns in sperm, was used to measure and analyze beat parameters using high speed image sequences filmed under dark-field microscopy (Hansen et al., 2018). The software capacity to track sperm head accuracy gives precise measurements on flagellar beat frequency and mean head velocity parameters. By analyzing parameters under the different divalent ion conditions, inferences were made regarding sperm motility. This approach can potentially lead to contraceptive development to control mosquito populations by testing different compounds that inhibit sperm motility. Further, understanding the molecular mechanisms that contribute to controlling sperm motility in

mosquitos can be a gateway that provides valuable information to other taxa, potentially even humans. This is made possible since many aspects of sperm biology, including the eukaryotic flagellum, are evolutionarily conserved across Animalia.

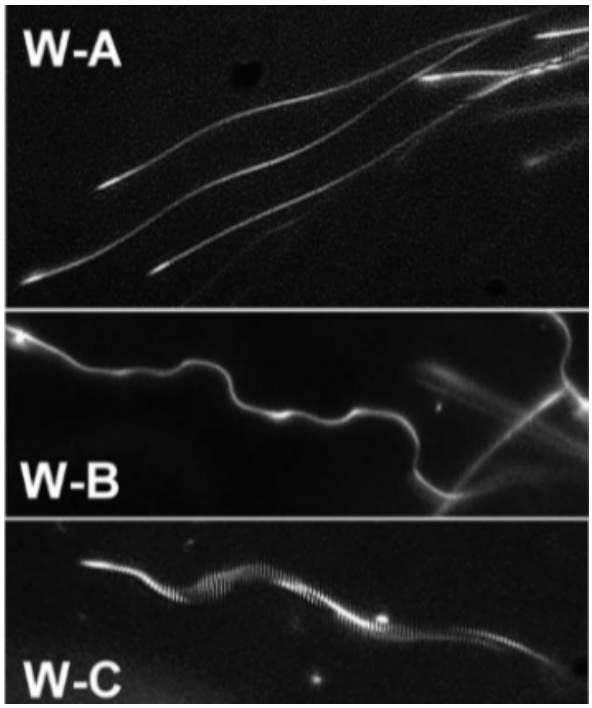


Figure 1. Three different waveforms (A ,B,and C) of *Culex quinquefasciatus* sperm. *Reprinted from "Waveform Generation is Controlled by Phosphorylation and Swimming Detection Controlled by Ca^{2+} in sperm from Mosquito Culex Quinquefasciatus" (Thaler et al., 2013).*

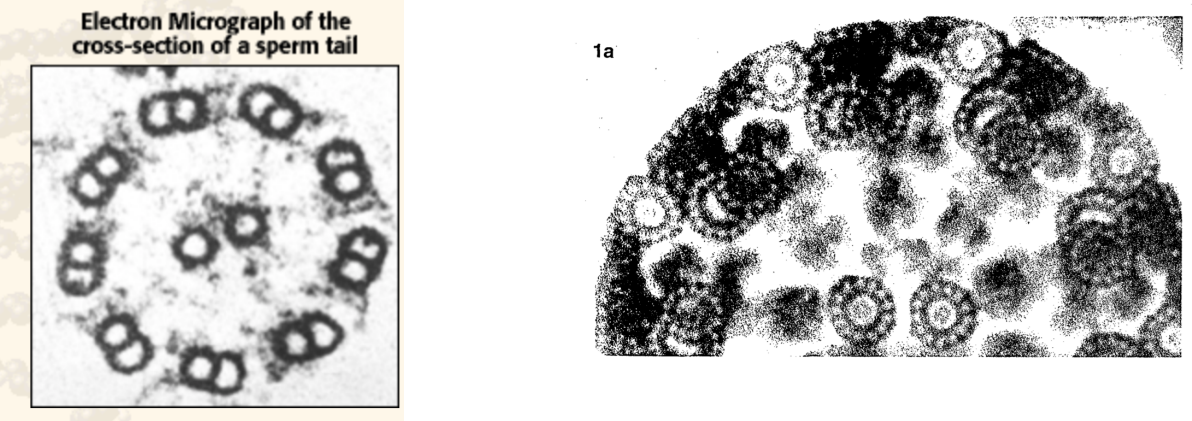


Figure 2: Diagram of the common "9+2" eukaryotic axoneme found in sperm. (Left). *Reprinted from "Axonemal structure and insect phylogeny" (Afzelius et al., 2009)*. Diagram of "9+9+2" pattern of insect sperm (Right) *Reprinted from " the sperm tail axoneme studied at high magnification" (Afzelius 1993)*.

Materials and Methods:

Chemicals:

Staurosporine, U0126, A23187, genistein, calyculin A, and okadaic acid were obtained from Enzo. Trypsin, aprotinin, and EGTA were purchased from Sigma-Aldrich. FR180204 was purchased from Tocris Bioscience (R&D Systems; Emeryville, CA). All other reagents were obtained from Sigma-Aldrich (Burlington, MA) or Fisher Scientific (Waltham, Massachusetts) (Thaler et al., 2013).

Insect Ringer's solution:

Insect Ringer's solution contains 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 7.4 at room temperature (Thaler et al., 2013).

Nickel and Zinc Ringer Solution:

Starting concentration for Nickel contained 50mM diluted 1:50 in 2x insect Ringer (2 times the concentration) with $NiCl_2$. The starting solution for Zinc contained 50mM diluted 1:50 in 2x insect Ringer (2 times the concentration) with $ZnCl_2$. Therefore, the working concentration of both was 1mM.

Imaging Equipment:

The sperm seminal vesicles/accessory glands with Ringer solution containing trypsin are placed on the microscope slide and transferred to microscope stage (Alphaphot; Nikon, Melville, NY). The Nikon microscope with darkfield condenser is used to increase contrast making sperm appear more brightly lit against a dark background. Images are recorded using Edgertronic SC1 high frame rate cameras. The computer containing ImageJ and SpermQ software was Dell, Precision 7920 Tower Model (Texas).

Imaging Software:

Edgertronic software was used for video capture. The recordings were used to quantify the timing of activation and changes in waveform. Sequences of sperm motility including overall progressive movement and flagellar beat patterns were obtained through the camera as well. Darkfield images using a 10X objective were taken at a frame rate of 200frame/sec or 100 frame/sec for ten seconds. To gain a better overview of all the sperm on the slide, the slide is moved three times so that three different fields of view are available. Through the camera recording an avi. movie file was created. ImageJ (National Institutes of Health) an Image processing program was used to take these avi. movie files and create stack tiff files otherwise known as frames. ImageJ calibrated the digital pixel distance value into microns. Images were prepared for analysis by adjusting brightness, contrast, threshold and background. SpermQ, simple analysis software used the processed images from Image J and quantified sperm motility parameters. Sperm Q provides a detailed quantification of the flagellar beat based on common

time-lapse images acquired by dark-field or epi-fluorescence microscopy (Hansen et al., 2018). To calculate the sperm head velocity, recording of the motility for the edgertronic camera was used to identify sperm moving in straight line trajectories and the velocity was calculated by measuring the distance traveled over 100 frames using Image J software.

Methods:

Maintenance of Mosquitoes:

Culex pipiens have a 16:8 light:dark cycle. Through the adult colony of mosquitoes reproduction of eggs/larvae are produced. Larvae are fed mouse chow with fish oil. Pupae are collected and placed into the cages for four days to allow the adults to emerge. Oviposition trays are set up in the cages using pupae water to attract females to lay eggs. Eggs are collected and put into cups with water, allowed to hatch, and added into pans with food. A vacuum aspirator was used to collect mosquitos.

Sperm Isolation and Activation:

Male mosquitoes were anesthetized using CO_2 and then euthanized using chloroform. The male reproductive tract consisting of the paired testes, seminal vesicles, and accessory glands were carefully removed from the abdomen and placed onto a microscope slide with Insect Ringer's at room temperature. Sperm were extruded from accessory glands and were activated by Ringers containing 2µg/mL Trypsin. The coverslip was placed on top of the slide with modeling clay on all four corners.

Converting images in Image J for sperm motility analysis

Image J was used to convert each ten-second movie into 1000 digitized frames using Image J. Using a stage micrometer, and using the magnification that was used for this study, I determined that 1 pixel was equivalent to 1.16 µm. Within Image J, regions of interest (ROIs) were identified which decreased the time of processing. Images were prepared for analysis by adjusting the brightness and contrast (Lin, 2021). After duplicating the image in ImageJ and selecting the frames you want to use (ex: frames 1-20) SpermQ Preparator can be used to perform Gaussian Blur to modulate the sigma size and reduce the noise in the image. Then, subtracting background modulates the pixel size. A small pixel size helps equalize intensity differences between head and tail of the sperm cell. After these steps SpermQ determines a rough flagellum structure using the skeleton method. Select “Plugins”, “Skeleton” and “Skeletonize" in ImageJ. This way you can see what is detected as a skeleton (Hansen, 2020). The specific steps that were used to optimize image quality can be found in Figure 1.1.

Quantitative Analysis of Sperm Motility Parameters

SpermQ was used to trace mosquito sperm flagella frame-by frame and various motility parameters were determined. SpermQ tracks the brightest points on the sperm head in each of the frames creating a trace of the distance traveled. The specific steps that were used to operate SpermQ to obtain the different parameters can be found in Figure 1.2.

Determining changes in sperm motility parameters in the presence of Calcium channel blockers

The first experiment was the control experiment. Sperm seminal vesicles/accessory glands with Ringer solution containing 2μg/mL Trypsin were placed on microscope slides and transferred to a microscope stage. The Nikon microscope and darkfield condenser was used to view the sperm. Sperm movements were then recorded using the Edgertronic camera. Darkfield images using a 10X objective were taken at a frame rate of 200frame/sec or 100 frame/sec for ten seconds. Through the recording an avi. movie file was created. ImageJ then converts it into 1000 frames. After adjusting the brightness and contrast in the SpermQ Preparator, SpermQ evaluator quantifies the sperm parameters like Mean Head Velocity and Beat Frequency.

The second experiment involved sperm seminal vesicles/accessory glands with Ringer solution containing 2μg/mL Trypsin + 1 mM Nickel which were placed on microscope slides and transferred to a microscope stage. Once again the Nikon microscope, Edertronic camera, ImageJ, SpermQ Perpator and SpermQ Evaluator were used to quantify the sperm parameters.

The third experiment involved sperm seminal vesicles/accessory glands with Ringer solution containing 2μg/mL Trypsin + 1mM Zinc which were placed on microscope slides and transferred to a microscope stage. Once again the Nikon microscope, Edertronic camera, ImageJ, SpermQ Perpator and SpermQ Evaluator were used to quantify the sperm parameters.

After, to compare the different parameters found in each experiment SigmaPlot was used. Through SigmaPlot, box-and-whisker plots and scatter plots were created to visually see the similarities and differences in Mean Head Velocity and Beat Frequency based on the different Ringer solutions used on each of the sperm samples.

Statistical Analysis

Data were analyzed using the Student's paired T-test, an inferential statistic, to determine if there was a significant difference between the means and medians of total velocity for control, zinc and nickel conditions. This would indicate whether the results were based on random chance or not.

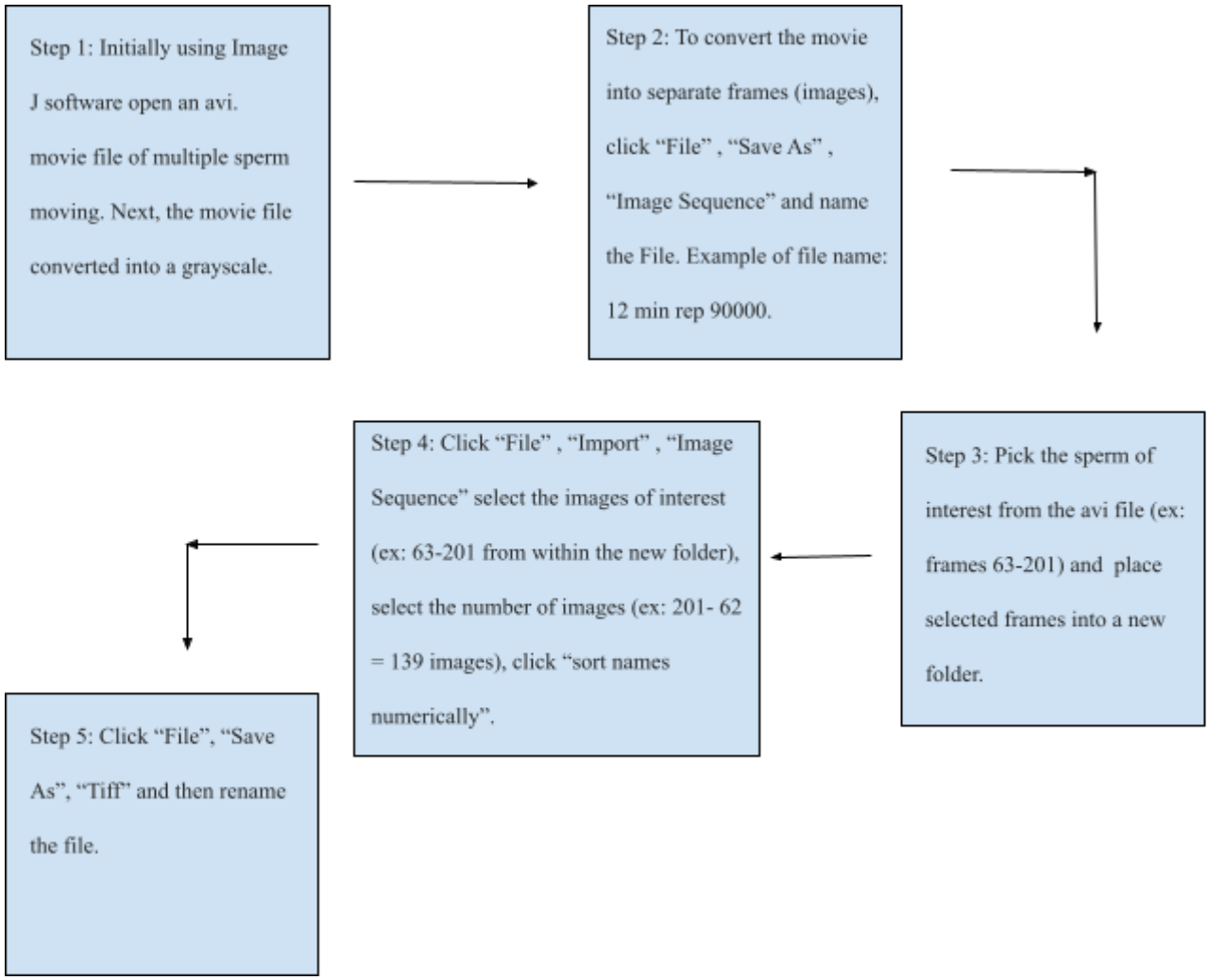


Figure 1.1: Steps on how Image J software analyze sperm motility

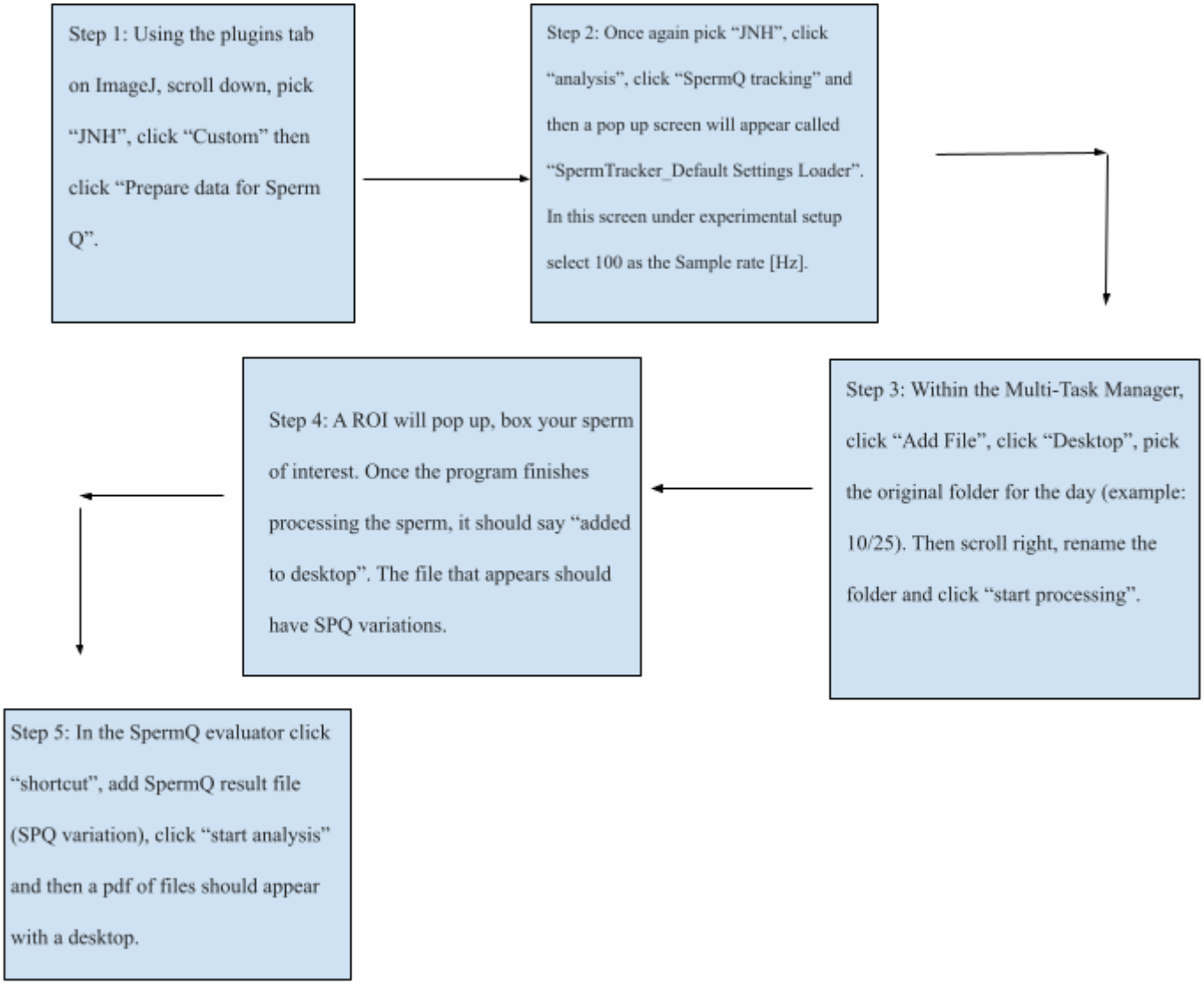


Figure 1.2: Steps on how to operate Sperm Q to analyze sperm movement

Results

The focus of this study was the impact of divalent cations Nickel (Ni2+) and Zinc (Zn2+) on sperm motility. The research question is: Do they have the potential to inhibit extracellular calcium channels of the sperm that would ultimately affect the downstream phosphorylation of flagellar proteins overall reducing sperm motility? This study takes a closer look at Mean Head Velocity and Flagellar Beat Frequency parameters. The Mean Head Velocity is the directional speed of a sperm head in motion as an indication of its rate of change in position as observed from a particular frame of reference and measured by a particular standard of time. Flagellar Beat Frequency is calculated by tracking the number of turning points of curvature for a choice of s, dividing by the time period T and then halving (Gallagher, 2019).

Three experiments were conducted to test this research question. The first was the control. In the control, the microscope slide had sperm seminal vesicle/accessory glands with Ringer solution containing 2μg/mL Trypsin. A Nikon microscope was used to view the sperm and an edgertronic camera was used to record the sperm movement, and saved in an avi.movie file. To produce quantitative parameters of the isolated sperm from the avi.movie file, Dark field microscopy, Image J, and SpermQ image processing were used. Dark field microscopy helps the sperm stand out from its black background (Figure 1.3). Image J is a software that converts the video recorded of the sperm into frame-by-frame pictures for further analysis. SpermQ is a software that uses the frames from Image J as input and converts them into quantitative parameters. The SpermQ Preparator adjusts the brightness and contrast to make the sperm more visible and then the SpermQ Evaluator produces the output values of the two parameters. This same procedure is repeated for the second and third experiment with one modification. The

Ringer solution contains 2µg/mL Trypsin + 1mM Nickel in the second experiment and 2µg/mL Trypsin + 1 mM Zinc for the third experiment.

Thus, three data sets were created by SpermQ. The first data set represents the control- activation of the sperm with Trypsin only. The second data set represents the second experiment- the activation of the sperm with Trypsin and Nickel. The third data represents the third experiment- the activation of the sperm with Trypsin and Zinc.

To analyze the data, the outputs were placed into SigmaPlot. Graphs like box-and-whisker plots and scatter plots were created to compare the results. In descriptive statistics, a box plot or boxplot (also known as box and whisker plot) is a type of chart often used in exploratory data analysis. Box plots visually show the distribution of numerical data and skewness through displaying the data quartiles (or percentiles) and averages. Box plots show the five-number summary of a set of data: including the minimum score, first (lower) quartile, median, third (upper) quartile, and maximum score (McLeod, 2019). A scatterplot shows the relationship between two quantitative variables measured for the same individuals (Mindrila). Then, paired t-tests were used to test whether the mean difference between pairs of measurements is zero or not (JMP Statistical Discovery LLC, 2022).

The parameters generated from SpermQ were then compared. For example, Figure 2 shows what general types of outputs were produced through SpermQ. Information on tracing Head position of the sperm in space as it moves a particular distance was quantified. The Flagellar Beat Frequency and Mean Head Velocity numerical values were also calculated and displayed by the software. Later, analysis of graphs were made comparing the Beat Frequency and Average Head Velocity of sperm under the three different experimental conditions.

Through the multiple Head position space graphs outputted from SpermQ software, the total velocities were calculated from the control, zinc and nickel experiments. The total velocity was calculated through dividing the distance it takes for the sperm head to move from one point to a second point in a given amount of time. For example, Figure 3 shows the Head position in space graph for zinc controlled sperm. To calculate the total velocity we divided 11.0 μm (the distance the sperm moved) by the time (1.04 sec). The total velocity for the zinc conditioned sperm is 10.5 μm/sec. After calculating the total velocity for multiple sperm in different experimental conditions a box and whisker plot (figure 4) was created to visually see the similarities and differences in the total velocity of each of the sperm in different experimental conditions.

For the control, the median total velocity was 50 μm/sec. The average total velocity was 51 μm/sec. There were some outliers like the 1 μm/sec total velocity, 120 μm/sec total velocity. In the zinc trails, the median total velocity is 9 μm/sec and mean total velocity is 13 μm/sec. These values are close together. There isn't much difference or variability. There is only one outlier that is 20 μm/sec in total velocity. In the nickel trails, there was a higher range in variability. Median total velocity is 70 μm/sec and the average total velocity is 80 μm/sec. There is the greatest variability in the nickel plot. With two outliers 190 μm/sec and 12 μm/sec. The highest median and average total velocities are in the nickel while the lowest are in the zinc. This may show that when nickel ions are used the sperm have a more total velocity and the sperm move faster given some amount of time. But with zinc ion the sperm have a smaller total velocity and the sperm move even slower than the control.

A t-test was conducted between the control and the zinc. The difference in the medium values between the two groups is greater than what would be expected by chance therefore there

is a statistically significant difference. This was also seen between the control and the nickel. But when comparing between nickel and zinc it was seen that there was no significant difference. Overall this shows that the results produced were not based on random chance. This makes sense because Nickel and Iron act as blockers of Ca^{2+} ion channels so they should have an impact on sperm motility that is not influenced by chance.

Next,the Average Head Velocity parameter and Beat Frequency parameter displaced in SpermQ was compared to see if there was a correlation between how fast the sperm beated impacted its velocity or speed of trajectory. Figure 5 shows a scatterplot comparing these two variables- the Average Velocity vs the Beat Frequency for the three experiments (Control , Nickel, and Zinc). The scatter plot showed that with the Control experiment, the higher beat frequency of 50 Hz results in lower Average Velocities consistently. But with lower beat frequencies the average velocity varies from having both high and low numbers. In the case of both cations (nickel and zinc) significant decreases in both parameters relative to controls was observed. This showed that zinc and nickel do play a role in reducing sperm motility. They act as inhibitors of Ca^{2+} entry, impacting the downstream phosphorylation of flagellar proteins.

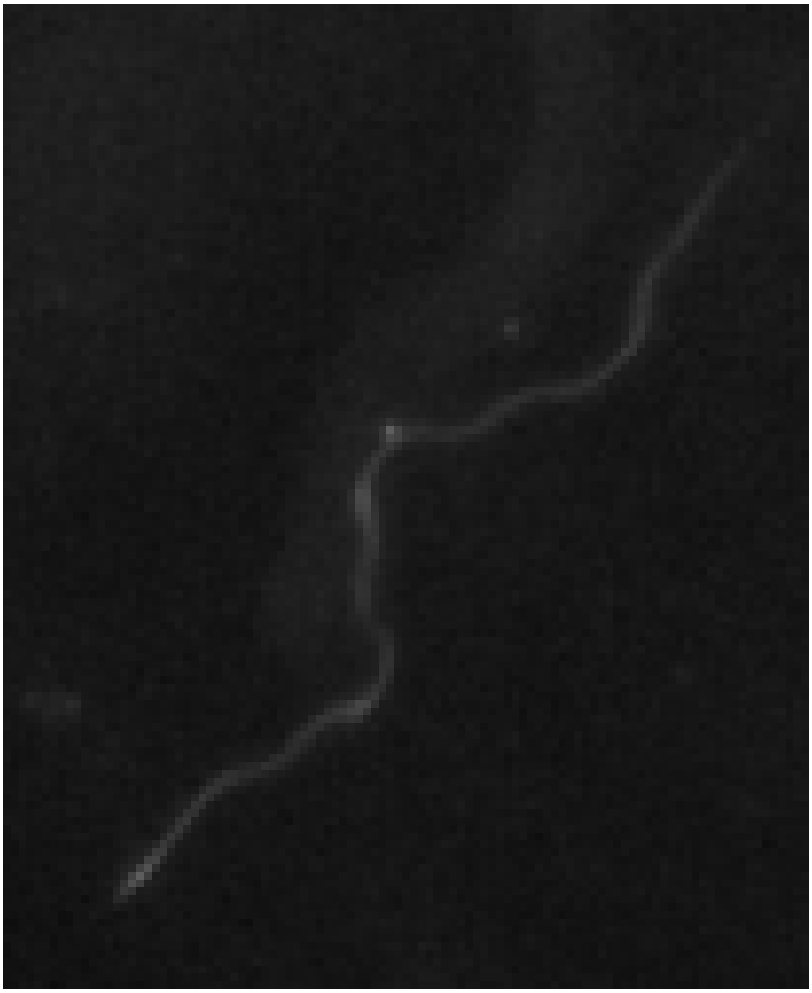
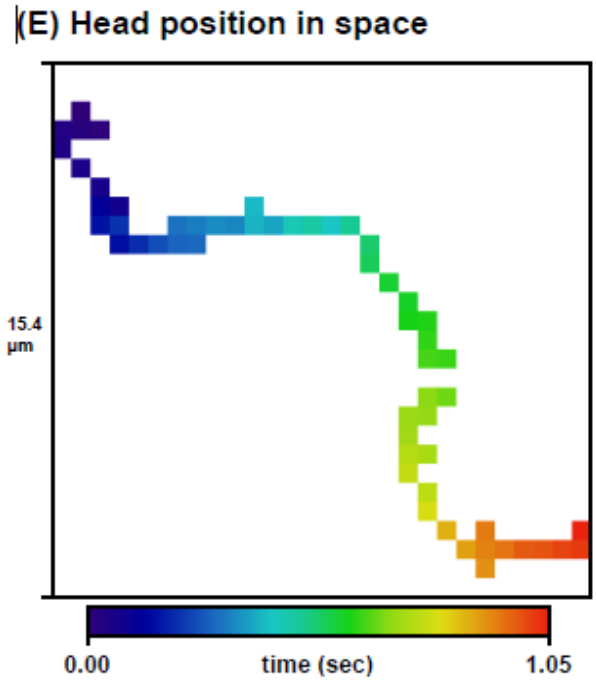


Figure 1.3: Example of the Sperm used. Raw image of a single mosquito sperm taken from Dark Field Microscopy.



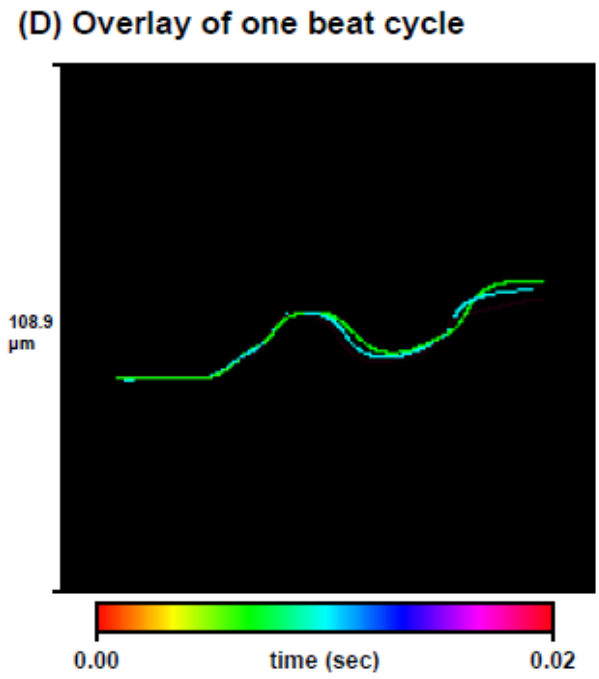


Figure A

Figure B

Figure 2: Control Experiment Data: Figure A indicates the location of sperm head over time. The Blue color is the starting point of the sperm and the red color indicates the end point of the sperm head. Overall the Red-Violet bar displays a timeline. Figure B shows the beat cycle of the sperm. One beat cycle: 0.02 sec. The length of sperm is 108.9µm long. The Beat Frequency: 50beats/sec and the Mean Head Velocity: 0.45 µm/sec.

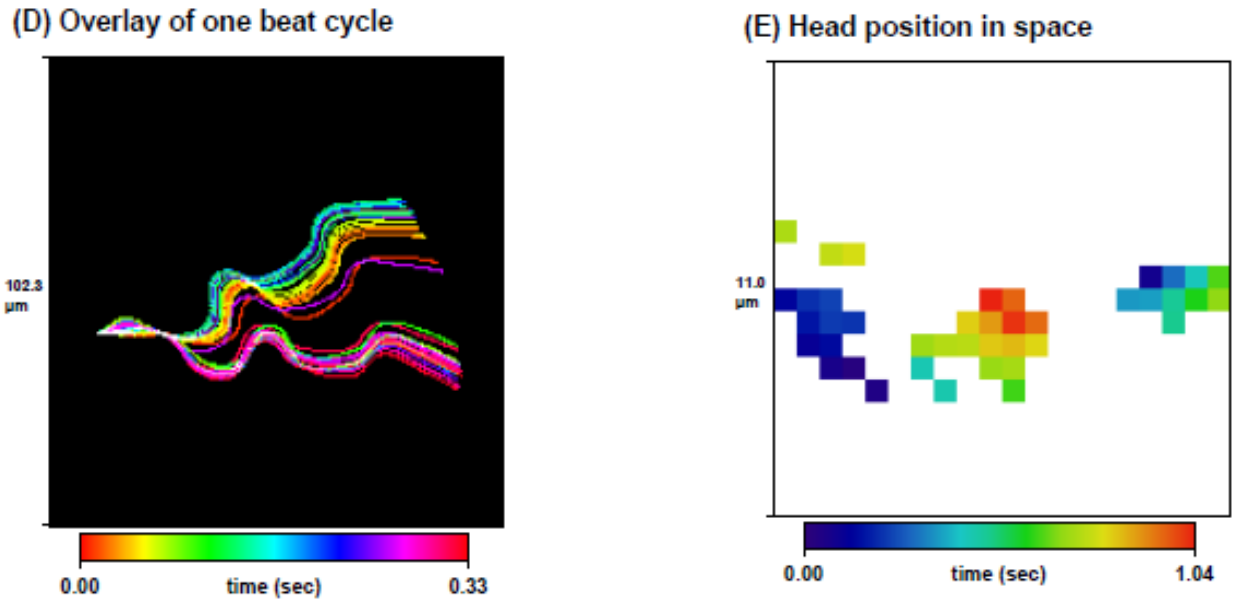


Figure 3: For the Zinc conditioned sperm the two figures indicate the beat cycle and the head position through space. The beat Frequency for the zinc treated sperm is 3 beats/sec and the Head Velocity is 1.92 µm/sec.

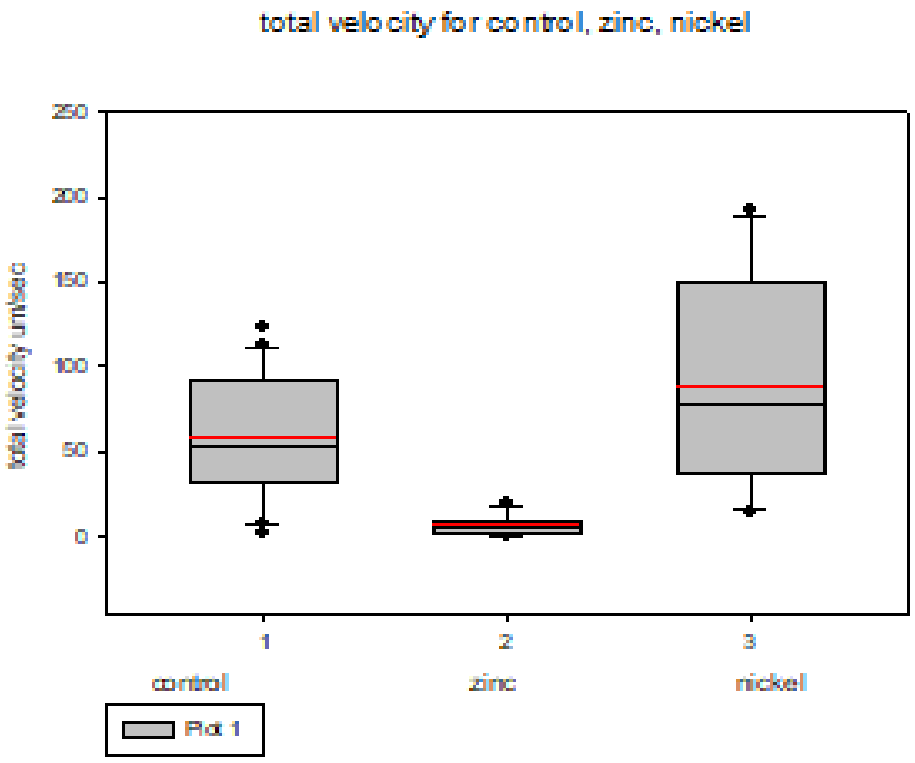


Figure 4: Box-and-Whisker Plot used to compare the total velocity for each of the three experiments (Control, Nickel and Zinc treated sperm). The Red lines indicate the mean total velocity and the dots represent the outliers. The middle black line is the median line.

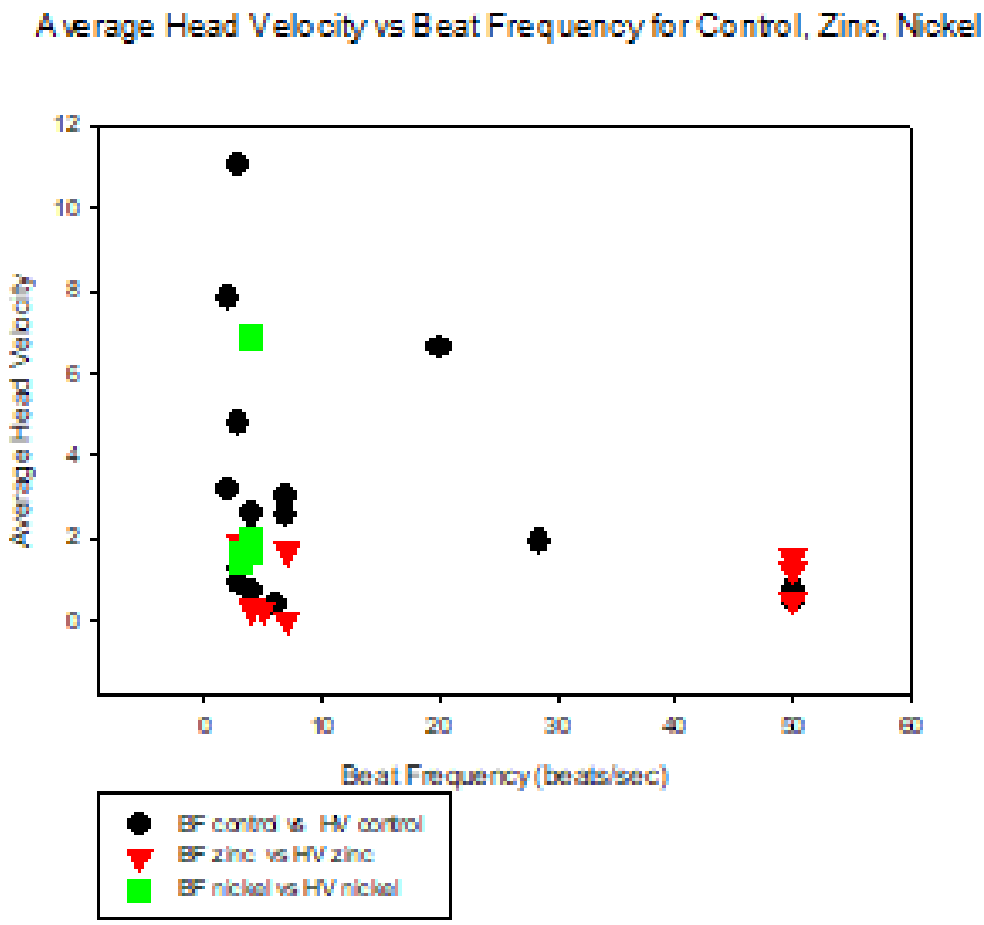


Figure 5: Scatterplot comparing The Average Head Velocity and Beat Frequency for the three different experimental conditions of sperm (Control, Nickel and Zinc).

Discussion

Previous studies on *Culex quinquefasciatus* showed that following activation, sperm progress through three distinct flagellar waveform patterns resulting in increased velocity and progressive motility. These would be waveform A, B and C. Trypsin protease activates the MAPK pathway and the MAPK activation is needed to generate waveform C. When accessory glands/seminal vesicles are activated with trypsin and Ringer solution containing Ca^{2+} the sperm is able to progress through all three waveforms. But in the absence of Ca^{2+} sperm remained immotile. When EGTA/okadaic acid substances were added and calcium was removed from the extracellular environment using calcium chelator, the sperm began to move backward (Thaler et al., 2013). Throughout the prior research Ca^{2+} was indicated to be a vital part initiation and maintenance of flagellar motility in mosquito sperm. But in this experiment we wanted to know whether the voltage gated calcium ion channels are required for the entry of the extracellular Ca^{2+} into sperm to promote sperm motility. Zinc and Nickel are known to be inhibitors of calcium gated ion channels that is why they are used in this experiment. Their impact on sperm motility would indicate whether a voltage gated calcium ion channel is necessary for the influx of extracellular Ca^{2+} lead to higher sperm motility. So the overall question we are trying to ask is whether Zinc and Nickel have an impact on sperm motility.

From the three experiments conducted, different parameters of mosquito sperm motility were measured. The parameters include Mean Head Velocity and Flagellar Beat Frequency. We looked at Mead Head Velocity as it shows how fast the sperm moves forward in a particular distance in a given time frame. This gives an accurate description of whether there is an increase or decrease in sperm motility. The larger the number for the Mean Head Velocity is, the faster the sperm is moving therefore the larger the sperm motility.

Some of the flagellar parameters looked at in this study included Velocity and Beat Frequency. The velocity measured how fast the sperm moves forward. The Flagellar Beat Frequency is the number of beats per second, equal to the difference in the frequencies of two interacting tones or oscillations. Both of these parameters indicate how fast a sperm would be moving overall giving an idea of the sperm motility and speed. SpermQ software is used on multiple types of sperm tracing sperm flagella frame-by frame calculating various motility parameters were determined. SpermQ tracks the brightest points on the sperm head in each of the frames creating a trace of the distance traveled.

The parameters indicated that Zinc can effectively reduce sperm motility by acting as an extracellular calcium channel blocker in the sperm. This is shown in Figure 4 where Zinc reduced the total velocity of the sperm. The Average Head Velocity and Beat Frequency also are shown at low values in Figure 5. Therefore, Ca^{2+} channel might be a good target and subsequent work could design mosquito specific drugs to block the channel.

The same cannot be said for Nickel. More studies need to be conducted on Nickel as this study shows mixed results on its ability to promote or inhibit sperm motility. Figure 4 indicates that Nickel increases total velocity and therefore implying an increase in sperm motility. Nickel has variability. Figure 5 states that Beat Frequency of Nickel induced sperm is 5beats/sec and the average head velocity is found to vary from 2 to 8 μm/sec which is much lower than the control this implied that Nickel might reduce motility. Regardless, more research is needed to clarify whether Nickel will inhibit or promote motility.

Getting an average flagellar amplitude parameter from spermQ was difficult. This would have been beneficial to consider when making final determinations on divalent cations' ability to impact sperm motility. Additionally, it was also hard to precisely track the head by only

focusing on the high intensity center mass in the head, because the helical beating pattern of the flagellum would cause the tail to move in and out of focus creating a high intensity brightness as the head. It would have also been beneficial to do more background research on different cations and their pathway in impacting the extracellular calcium channels. Zn and Ni are known to block calcium voltage gated ion channels. As such, they can influence the entry of Ca^{2+} into the channels impacting the sperm motility. In particular they would block these channels causing no Ca^{2+} to enter, reducing the sperm motility in theory. The experiment showed this in practice and sperm with Zinc induced ringer did show reduced sperm motility.

It has been shown in humans that Zinc is an essential element for male fertility. Zinc as a hormone balancer helps hormones such as testosterone, prostate and sexual health and functions as an antibacterial agent in men's urea system. It plays a role in epithelial integrity, showing that Zn is essential for maintaining the lining of the reproductive organs. Zn deficiency impedes spermatogenesis and is a reason for sperm abnormalities and has a negative effect on serum testosterone concentration (Fallah et al., 2018). As sperm activation stays consistent among animal systems it makes sense that Zinc deficiencies would cause the same outcome in both humans and mosquitoes. The follow up question would be trying to understand if its possible to have zinc in its gaseous form be distributed through the atmosphere. This way it's impacting the adult mosquitoes already in the population. Previous research has shown that zinc oxide nanoparticles using plant *Lawsonia Inermis* is effective in controlling targeted mosquitos (Amuthavalli et al., 2021).

Although this sounds promising in theory, large concentrations of zinc in the atmosphere can be toxic. Zinc oxide would impact the other species in the habitat leading to unwanted deaths. Other animals and humans have Ca^{2+} voltage gated channels as well. The zinc oxide can

inhibit the channels in these other species causing reduced survival rates of different species. Using zinc oxide would kill the mosquitos but at the same time would also potentially kill off other living things living in the same environment as mosquitos. This would defeat the purpose of using zinc oxide. Therefore a more beneficial solution would be to find an inhibitor of Ca^{2+} channel specifically to mosquitoes. So yes, knowing that zinc is a good inhibitor Ca^{2+} voltage gated ion channels is a good first step in controlling mosquito populations in a controlled lab setting but not necessarily in real life. A unique inhibitor effective in only inhibiting specific channels in mosquitoes needs to be found.

Overall, this study showed that sperm motility is inhibited by Zn^{2+} suggesting that the Ca^{2+} voltage gated channels are required for the entry of Ca^{2+} into sperm. Without the entry of Ca^{2+} sperm motility will be hindered. A logical next step would be to improve the measurements on Average Head Velocity and Beat Frequency while also finding the average value for amplitude through SpermQ analysis. These would all give better indication and accurate data of zinc effect on motility.

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