MEASUREMENT OF ECDYSIS TRIGGERING HORMONE (ETH) AND ITS RECEPTOR LEVELS TO DETERMINE FUNCTION

By

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ABSTRACT

Mosquitoes, insects from the order Diptera, are a commonly known pest famous for not only being a nuisance to humans, but as well as serving as vectors to deadly diseases to humans all across the world. A mosquito's life cycle in the wild lasts about 1-2 weeks, with the female mosquitoes outliving the males in order to fulfill their primary role, reproduction of the next generation of mosquitoes. As holometabolous insects, they undergo metamorphosis from an egg, to larva, to pupa, until they emerge as adults, and reproduce to begin the entire cycle over again. One vital hormone that is responsible for the progression of metamorphosis is ecdysis triggering hormone, also known as ETH for short. Although known solely for the transformation of insects, research has shown that ETH can also play a role in the reproduction of adult female mosquitoes. Immunohistochemistry was utilized to determine the location of the ETH receptors within the mosquito during specific time periods in their life cycle and analyze the activity level from each chosen stage. Mass spectrometry was then utilized to assess when concentration of ETH in the hemolymph of the mosquito is the highest. This study is hoped to provide insight on the importance of ETH on metamorphosis for mosquitos in regards to their physiology, as well as its association with other prominent hormones used for development.

Acknowledgements

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Introduction

Mosquitoes, also referred to by their scientific name, *Culicidae,* are a holometabolous insect from the order Diptera. They can be found in areas all around the world, except areas with really cold weather, and they feed on the nectar of flowers and plant sap. It is often presumed that mosquitoes also suck the blood out of humans, mammals, and other organisms as a source of nutrition, when in reality, it is only the females that obtain blood from their eggs and its solely to aid in egg production. Mosquitoes are most known to transfer harmful and deadly pathogens to humans and other animals, such as Zika virus, malaria, and yellow fever. Mosquitoes have a detailed life cycle, starting as an egg, to developing into a larva, a pupa, and finally an adult (Figure 1) and they respond to different environments depending on their developmental stage and the hormones involved. Although complex, the cycle is controlled by a single mechanism, hormone signaling. Hormone signaling allows for regulation of hormones that contribute to the development, growth, and reproduction of mosquitoes. Extensive research has been done on hormone signaling in similar organisms such as moths and the common house fly, however, little is known about this subject in mosquitoes. The focus of my capstone project is geared towards understanding the components of hormone signaling and what hormones have the biggest impact in certain developmental stages, as well as other implications certain hormones may have on other systems of the mosquitoes' body. Research will be conducted on the Ecdysis Triggering Hormone, ETH, as well as its receptors.

Background/Literature Review

My research is focused primarily on ETH and its receptors during female mosquito development. The reason for focusing on female mosquitoes is due to the nature that male mosquitoes' only essential role is to provide the sperm needed to fertilize the eggs. Unlike females, males do not contain the mouth parts necessary to extract blood from their hosts, therefore they can not serve as vectors for devastating diseases (National).

ETH is a specific peptide that is stored and released in the Inka cells of mosquitoes. This peptide plays a vital role in initiating the ecdysis, or growth, sequence of the mosquito (Roller). This hormone is found in all other insects that undergo some type of metamorphosis, however our experiments are conducted on *Aedes aegypti,* also known as the Yellow Fever mosquito, due to the further possible implications that can be made on this specific species of mosquito and modifying its development. ETH is an essential hormone that allows for the mosquito to transform from egg to larva, larva to pupa, and eventually from pupa to a full grown adult mosquito. ETH itself is produced and released by Inka cells (Figure 2) , but the synthesis and release of this hormone, along with the expression of its receptor, is regulated by ecdysone, a steroid hormone that is produced by the prothoracic gland of the insect (Meiselman 2017). Ecdysis triggering hormone has a high impact on the central nervous system, creating a signaling cascade when the mosquito is going to undergo a stage of ecdysis, which occurs during every instar, a total of four in the mosquito (Dai). Other studies that have been conducted on different insect organisms, like *Drosophila,* demonstrated that ETH was vital towards the endocrine system, and the absence of the *eth* gene was lethal to the insect, causing severe deformities and death after the first ecdysis (Zitnan). It has been shown that ETH has a variable effect in the amount of eggs a female mosquito produces and releases, depending on the type of environment

it is exposed to, such as heat or nutrient stressed environments, can cause a decrease in egg production (Meiselman). These results led us to hypothesize that and investigate the potential roles of ETH in the physiology of the mosquito besides being essential for initiating the process of ecdysis. A study conducted in 2006, which consisted of ETH having effect in the type of behavior displayed by *Drosophila,* supports our theory (Kim).

Along with this, the other focus in our project is to determine the relationship that ETH levels have with two other hormones that are present during ecdysis, Juvenile hormone and ecdysone. Juvenile hormone assists in regulation of the reproductive properties of the mosquito and assists ETH in determining the appropriate time frame to initiate development and release of the eggs (Goodman). Ecdysone contributes to the quality of the eggs and the amount that is released (Riddiford). ETH is known to help regulate the biosynthesis of juvenile hormone, how much is produced, and when it is to be released into the hemolymph in the mosquito (Arieza). This is due to the fact that Juvenile hormone is also required for the processes that control the post eclosion development of the midgut, fat body, and ovaries. These organs are responsible for the digestion of the blood meal to obtain the proteins that will be utilized in reproduction (Arieza). The reasoning as to why we are choosing to focus only on mosquitoes is because various studies have been conducted on similar topics using flies, and have resulted in promising results (Park). Specific research on mosquitoes may allow us to reveal if there are any smaller differences in the purpose of ETH between flies and mosquitoes, being that there are very distinct differences between the two organisms themselves.

Understanding how each different hormone works on its own, as well as how they all work together and contribute to the growth and the development of the mosquito, is essential for future applications towards disease control on the pathogens that mosquitoes transmit. Our

research, if successful, can expand our knowledge on what contributes to certain growth stages of mosquitoes, and if possible, be able to modify the growth stages to certain results that can affect how mosquitoes can transmit pathogens. The plan is not to limit or decrease the population of mosquitoes in the ecosystems all around the world, but to possibly modify the genotype and evolution of the mosquitoes to eliminate their capability of carrying disease vectors. With this research, the hope is to reduce and/or possibly eliminate the deadly diseases that these organisms carry.

Figures/Diagrams

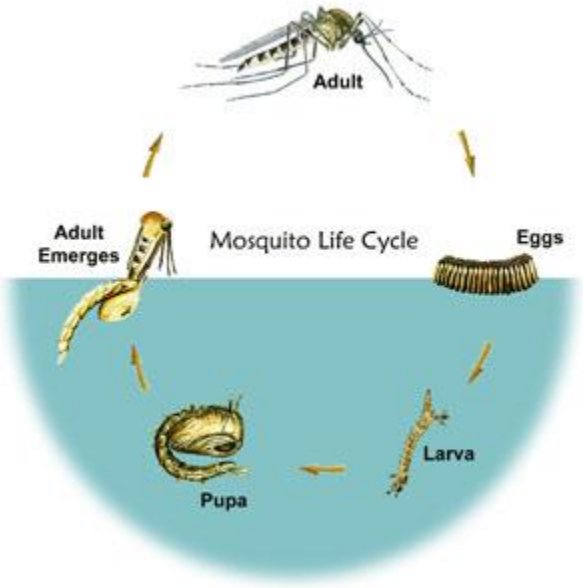
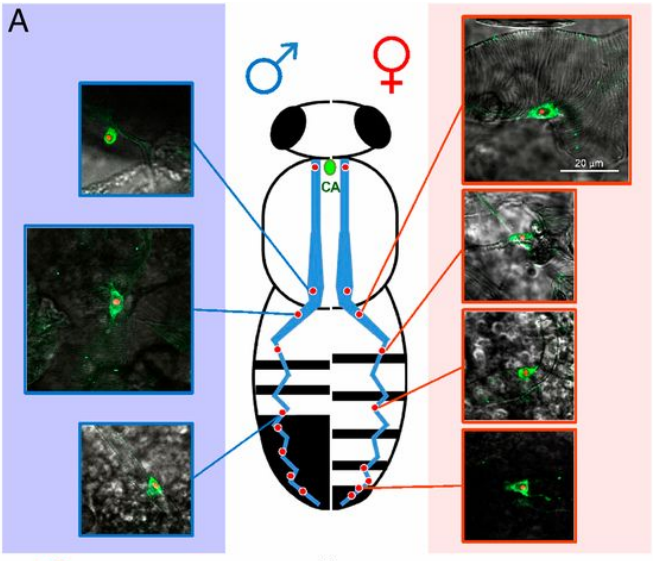


Figure 1: Mosquito Life Cycle

The female mosquito only reproduces after receiving a blood meal. The first three stages of life for a mosquito occurs in the water, so the female adult lays her eggs either on or near a water source, or in an area such as soil where water will be accessible. Once laid, eggs then hatch into larva, and the time it takes for an egg is variable between species, and is also affected by external factors such water temperature and food availability. Larva continues to live in the water, where it feeds and molts to develop into the next stage, pupa. In its pupal form, it remains in the water, but it no longer feeds, and remains in its pupal form for about two days to about a week, until it emerges from its pupal case as an adult and repeats the cycle over again. As mentioned before, depending on the species of the mosquito, as well as external environmental factors, the total length of time for its life cycle can range from as quickly as 4 days, to as long as 1 month. (*EPA)*

Figure 2: Location of Inka Cells

The figure to the right shows the location of the Inka cells in the mosquito, where ETH is produced and released from. They drove the expression of the RedStinger protein and performed immunostaining for ETH in order to visualize the Inka Cells. The left and right side of the diagram depict the location Inka cells were found along the tracheal systems of male and female drosophila (Meiselman 2017).



Application to Epidemiology

As stated previously, mosquitoes are most notorious for carrying deadly pathogens. From malaria, to Yellow fever and West Nile virus, these insects pose serious health risks, especially in underdeveloped countries where healthcare is not as advanced compared to first world countries. Specificity regarding transmission of disease is dependent on the species of the mosquito. More than one species can serve as a "messenger" for the same virus or parasite, while some vectors might only be viable in a specific species of mosquito due to their genetic composition (Mutebi). Mosquitoes are not responsible for creating the pathogen, but merely a way of passage from one host to another. The adult female mosquito must first receive a blood meal from an organism who contains the pathogen in their bloodstream. From there, when the same mosquito moves onto their next source of nutrients, another blood meal, the transmission to the new host occurs, and depending on the type of organism, the newly introduced vector can either have no harm, or raise havoc in its new host (Mutebi). The mosquito during the entirety of this is not affected by the pathogen they carry.

The focus of our research, *Aedes aegypti,* is one of the many species of mosquito that transmits Yellow fever. Yellow fever is a viral hemorrhagic fever caused by the Yellow Fever virus. Around 15% of people who are infected experience the more severe form of the disease (Mutebi), while the rest only experience mild symptoms such as fever, headaches, weakness, and recover fairly quickly, or do not have symptoms at all (CDC). For those that unfortunately were affected by the clinical symptoms, they undergo 2 stages of the virus, the period of infection and the toxic phase. During the period of infection, the infected human experiences the typical symptoms of the disease, and is infectious to mosquitos, meaning they can continue to infect other people if a mosquito feeds on an infected person (Mutebi). They then enter the

remission phase where their clinical symptoms disappear for 24 hours, and proceeding the remission phase they begin the toxic phase. During the toxic phase, the intensity of the symptoms increases drastically, and can cause severe complications that lead to death, or in rare cases, immunity for life if they are able to recover (Mutebi). Cases of Yellow fever are most common in Africa and South America, and travelers to these regions are advised to visit with precaution, and vaccinate themselves to protect oneself (CDC).

The connection between *Aedes aegypti* and its transmission of Yellow fever was made in the 20th century, however beginning in the 1930s and forward, there have been a total of 3 transmission cycles for the Yellow Fever virus that have been recognized in Africa. The three cycles: the jungle cycle, the intermediate cycle, and the urban cycle, are different from one another, but the connections to each other help increase the rate at which the virus is spread amongst a population (Figure 3) (Mutebi). The jungle cycle takes place primarily in the African forest where transmission occurs primarily through *Aedes africanus* and primates. Things shift into the intermediate cycle when a mosquito of *Aedes africanus* with the Yellow fever virus feeds on a human, or another mosquito from the *Aedes* genus feeds on a primate with the Yellow Fever virus. In the urban cycle, the pathogen is transmitted from human to human via *Aedes aegypti*, and it is during this cycle where the transmission of the disease is the most deadly due to how easily it spreads with the presence of a population in one certain location (Mutebi).

There is speculation that there are genetic differences between the different vectors of the virus. As the virus is in the mosquito, it is replicating and adapting to the genetic variation of the species it is in. This also includes any environmental or regional factors that the mosquitoes have to adapt to, leading to different genotypes of the same virus in different regions of Africa (Mutebi). It is believed that this potentially may have been the main factor contributing to the

evolution of the Yellow Fever virus across Africa, and how more research and work must be supplied in order to fully understand the evolution of the virus within the vector. With the virus continuous evolving, the focus is turned to whether there is something within a mosquito's genotype that can be modified to disrupt its ability to harbor and distribute the virus.

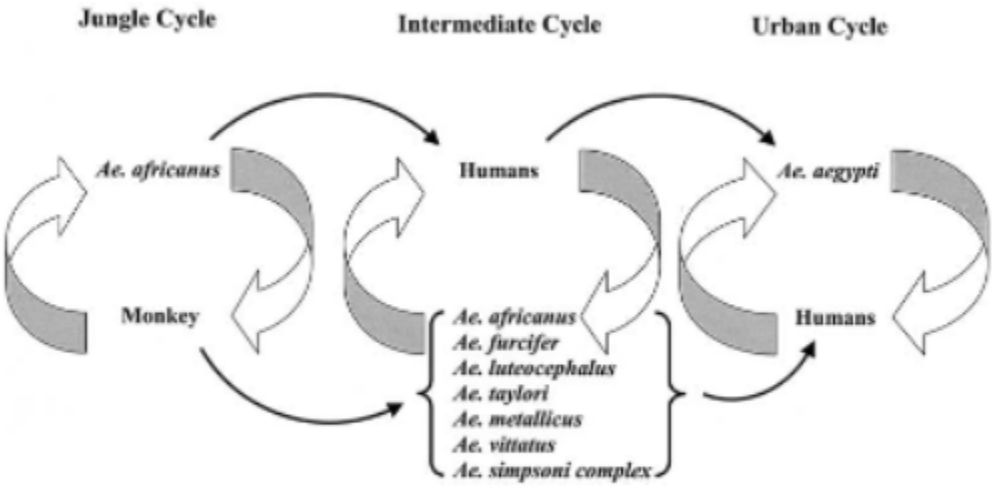


Fig. 4. YF transmission cycles in Africa.

Figure 3

This figure shows the connections between the three cycles and what components affect another.

Materials & Methods

The organisms used for this project are mosquitoes, species *Aedes aegypti.* Both Wild Type and Knockin were utilized. Eggs saved from the previous generation were used to produce the mosquitoes needed for immunohistochemistry and hemolymph retrieval.

Organism Growth

A small sheet of paper towel containing about 100 eggs was placed in a clear rectangular container filled halfway full with distilled water to allow eggs to hatch into larva (Figure 4). During the larval stage, mosquito larvae were given crushed dog food as their source of nutrition every other day for about 3-4 days until they became pupa. Once in pupal form, mosquito pupa were then transferred to small clear sauce containers via plastic pipettes, and placed in mosquito cages with a sugar water feeder, where they emerged from the water as full grown adults 3-4 days later. Pupas that needed to be obtained before eclosion for dissections were not placed in the mosquito cages and were set aside accordingly. Small amounts of adult females were utilized from every growth cycle to reproduce more eggs that could be stored for later use. Both immature and adult forms of the mosquito were kept in a room with a humidifier to maintain proper moisture in the environment and to provide an ideal environment for growth.

Immunohistochemistry

Immunohistochemistry was used in order to enhance the ETH receptors to make them more identifiable, and compare the locations of the receptors across various developmental stages to the stage with the highest receptor activity. To prepare the sample for immunohistochemistry, the mosquito (larval form, pupal form, and adult form) are dissected vertically on their ventral side

(Figure 5). Once the incision is made, a phosphate buffer solution (PBS) is added to the organism in the petri dish. PBS is used to prevent denaturation of proteins when the organism's body is exposed, as well as prevent a pH change when the body is exposed to air. Paraformaldehyde (PFA) is utilized to fix the tissues and prevent the skin from curling or folding onto itself, making visualization of the slides less difficult. The sample remains in PFA for 24 hours, and is then washed with a combination of PBS and PBST. Washing occurs within the span of 5 minutes, and is repeated 5 times to remove any excess PFA that is left on the body. Immunohistochemistry is now ready to be performed. Rabbit anti-GFP, the primary antibody, is used to ensure that the binding occurs on the specific ETH receptors we are looking for. To aid us in visualizing the receptors much easier, we utilized a secondary antibody, Alexa Fluor 488 anti-rabbit, which binds to the primary antibody, and once activated, emits a fluorescent color to increase the visibility of the receptors through the microscope. Immunohistochemistry is performed at the stage of the 4th instar (late pupa), late larva, and after blood feeding in adult females, stages where we believe that ETH receptor activity is at its highest.

Mass Spectrometry

Mass spectrometry was then used to identify the concentration of ETH in the hemolymph of the female mosquitoes. Fine needles were made from 100-µL micro-glass capillary tubes using a pipette puller P-30 (Figure 6). Needles were then inserted manually through the thoracic intersegmental membrane into the thoracic cavity, and insects were perfused with 20 µL of a “bleeding solution” of phosphate-buffered saline (PBS). The hemolymph was obtained from a small tear made laterally on the intersegmental membrane of the last abdominal segment. The first drop of the perfused hemolymph that was extracted was collected in small labeled glass

tubes (Figure 7), and stored in a freezer at -80 degrees Fahrenheit immediately after. Keeping the samples a very low temperature would prevent the denaturation of proteins in the hemolymph, and provide us with the most accurate measurement of ETH concentration. Our lab did not have the machinery/technology for mass spectroscopy to analyze the hemolymph, however we were able to collaborate with Drs. Francisco Lima and Cesar Rodriguez at Florida International University since they had the necessary equipment and materials to do so.

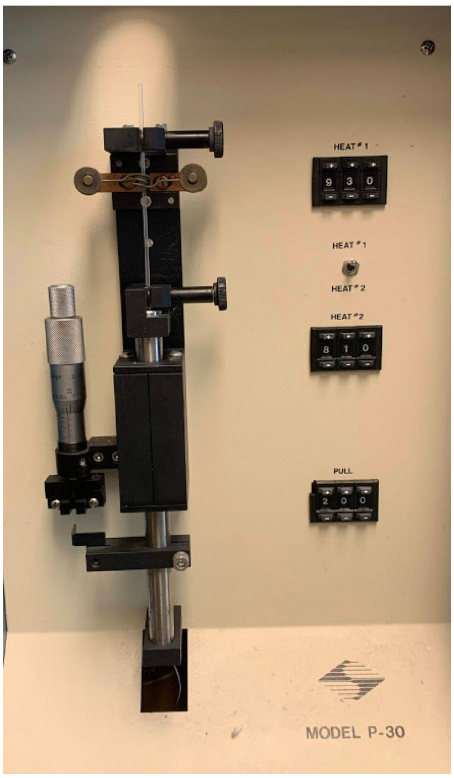
Figures

Figure 4 Recently hatched larva in clear containers. Once all eggs have been hatched, paper towel is then removed, and larvae are fed every other day until they transform into pupa. (Right Photo) Closer view of hatched eggs. Small black specks (red arrow) are the eggs that still have not hatched yet. Note that it may be possible that not all the eggs are guaranteed to hatch, hence why the amount of eggs placed are more than we plan to utilize. Blue arrow points to hatched larvae. As they mature into pupa, their shape changes from long, to a shorter, wider organism that resembles the shape of a comma.

Figure 5 View through microscope of dissected mosquito. Organs located on both sides of the medial line are the ovaries. When dissecting, two pins are used to stabilize the mosquito to facilitate smooth dissection, one inserted in the head of the mosquito, and one towards the end of the abdomen. Very sharp and small dissection scissors are used to create a small opening at the bottom of the thorax, and open up the thorax, and then proceed with dissecting the abdomen through the middle, ensuring that pressure used to slightly puncture the cuticle is not too much to avoid destroying any of the internal organs.

Figure 6

P-30 used to create the needles that were used to Inject the bleeding solution into the mosquito



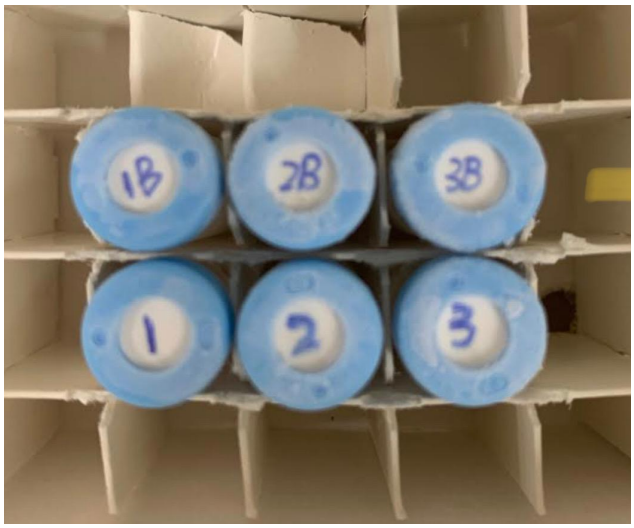


Figure 7

Labeled tubes containing the hemolymph and blood buffer used to extract it. Each tube contained samples of the hemolymph mixture from 15 mosquitoes in order to have a sufficient amount to be able to have an accurate reading from the machinery that would be used in Florida.

Results

Hemolymph was collected for the first round of ETH concentration analysis. A total of 6 samples were collected, tubes 1-3 each containing hemolymph from 15 non-bled female *Aedes*, and tubes 1a-3a each containing hemolymph from 15 female *Aedes* 24 hour after blood feeding. The samples sent to Florida International University for mass spectrometry returned with only Sample 1b showing readable levels of ETH in the hemolymph (Figure 8). The data provided was incomprehensible, however, this is the preliminary data that are being used to work out conditions for quantifying future ETH blood samples using an isotope-labeled internal standard.

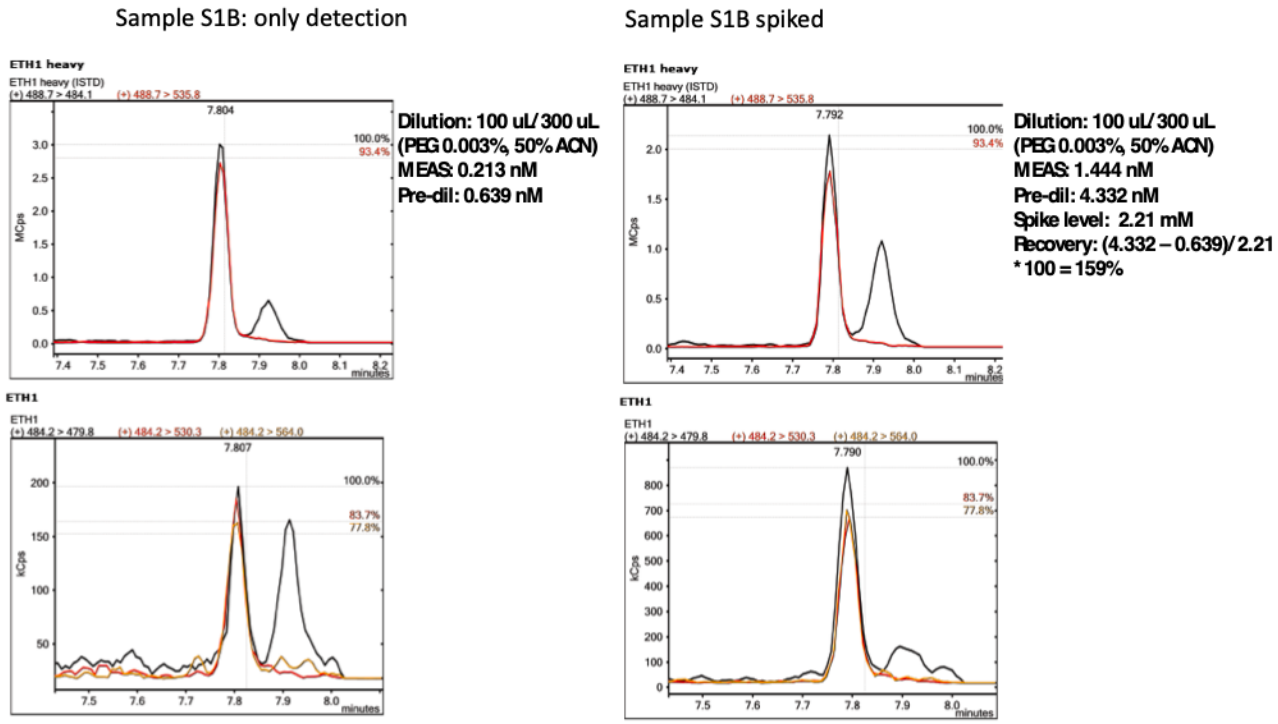


Figure 8: Mass spectrometry results from hemolymph collection.

Future Direction

Due to the unforeseen events of the COVID-19 pandemic, we were unable to continue to forth with our desired plans for our research. Only the first set of bleeding was able to be completed and sent before classes went remote and students were unable to be in the lab. Being that the majority of our research was hands-on, with raising, dissecting and bleeding the mosquitoes, it was very difficult to make any sort of progress to achieve results.

Nevertheless, I hope to continue with this research once students are allowed back on campus, for our work was just starting. Although the results did not provide us with the information we needed, we can still use our results to formulate our future experiments to obtain our desired results. Some of our experiment groups we planned to utilize in the future involved placing the mosquitoes in different types of environments that would induce stress. This stress would be induced by various factors, such varying habitat temperatures, as well as stress due to food availability. We are hoping these experiments would allow us to see if they cause any variation on the amount of ecdysis triggering hormone is released, and if it has any effect on the production of eggs in the female mosquitoes. We would also make sure to continue trying to obtain more results from our control group so we can construct our baseline data that we will use to compare to the variations of the experiments. Although the pandemic may have caused a major setback in our research, we want to look at it as an opportunity to better formulate our thought process and execution to obtain results that can further be applicable to disease control in mosquitoes.

References

Areiza, M., Nouzova, M., Rivera-Perez, C., & Noriega, F. G. (2014). Ecdysis triggering hormone ensures proper timing of juvenile hormone biosynthesis in pharate adult mosquitoes. *Insect Biochemistry and Molecular Biology*, *54*, 98–105.

Dai, L., & Adams, M. E. (2009). Ecdysis triggering hormone signaling in the yellow fever mosquito Aedes aegypti. *General and Comparative Endocrinology*, *162*(1), 43–51

Goodman, W.G., & Cusson M. (2012). The Juvenile Hormones. *Insect Endocrinology, 1*(8), 310-365

Kim, Y.-J., Žitňan, D., Galizia, C. G., Cho, K.-H., & Adams, M. E. (2006). A Command Chemical Triggers an Innate Behavior by Sequential Activation of Multiple Peptidergic Ensembles. *Current Biology*, *16*(14), 1395–1407.

Meiselman, Matthew, et al. "Endocrine Network Essential for Reproductive Success in Drosophila Melanogaster." *Proceedings of the National Academy of Sciences*, vol. 114, no. 19, 2017, doi:10.1073/pnas.1620760114.

Meiselman, M. R., Kingan, T. G., & Adams, M. E. (2018). Stress-induced reproductive arrest in Drosophila occurs through ETH deficiency-mediated suppression of oogenesis and ovulation. *BMC biology*, *16*(1), 18

"Mosquito Life Cycle." *EPA*, Environmental Protection Agency, 13 Mar. 2017, www.epa.gov/mosquitocontrol/mosquito-life-cycle.

Mosquitoes: National Geographic. (n.d.). Retrieved May 06, 2021, from https://www.nationalgeographic.com/animals/invertebrates/facts/mosquitoes

Mutebi, John-Paul, and Alan D.T Barrett. "The Epidemiology of Yellow Fever in Africa." *Microbes and Infection*, vol. 4, no. 14, Nov. 2002, pp. 1459–1468., doi:10.1016/s1286-4579(02)00028-x.

Park, Y., Kim, Y.-J., Dupriez, V., & Adams, M. E. (2003). Two Subtypes of Ecdysis-triggering Hormone Receptor inDrosophila melanogaster. *Journal of Biological Chemistry*, *278*(20), 17710–17715.

Riddiford, L. M. (1994). Cellular and Molecular Actions of Juvenile Hormone I. General Considerations and Premetamorphic Actions. *Advances in Insect Physiology Advances in Insect Physiology Volume 24*, 213–274.

"Yellow Fever Maps." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 15 Jan. 2019, www.cdc.gov/yellowfever/maps/index.html.

Žitňan D., et al. “Conservation of Ecdysis-Triggering Hormone Signalling in Insects.” *Journal of Experimental Biology*, vol. 206, no. 8, 2003, pp. 1275–1289., doi:10.1242/jeb.00261.